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Persistence of Neutralizing Antibody for a Year following Vaccination of Monkeys with Lansing Poliomyelitis Virus.* (18180)

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Whenever the question of vaccination against an infectious disease arises, two primary questions are asked. First, is there a safe, effective vaccine available; and second, is the immunity induced sufficiently lasting to achieve the desired result? For poliomyelitis, these questions are so far still under discussion as regards the experimental disease. A procedure for immunizing monkeys with active poliomyelitis viruses has been described (1), which however is not without risk to the experimental animal(2). After repeated intramuscular injections of sufficient doses of active virus, rhesus monkeys became immune to the extent that they showed high concentration of circulating antibody and resisted a large intracerebral dose of the same active virus. Furthermore, the level of circulating antibody associated with immunity to intracerebral resistance has been defined(3). The intracerebral resistance and the neutralizing antibody were found by cross testing with various types of poliomyelitis virus to be specific for the immunological type of poliomyelitis virus used for immunization(2,4,5). Having available an effective means for immunizing monkeys, the next important step was to find how long such immunity would last. Three rhesus monkeys which had been immunized with active Lansing poliomyelitis virus in the course of earlier work(3) were available for study of circulating antibody. After a course of intramuscular vaccination, all three individuals proved immune to intracerebral challenge with a large dose of Lansing virus. Following an additional intramuscular dose of virus, the monkeys were bled at two months' intervals for a year. At the end of the year, they received a single booster dose of virus intramuscularly, and

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* Aided by a grant from the National Foundation for Infantile Paralysis.

† Now Isabel Morgan Mountain, Westchester County Department of Laboratories and Research, Grasslands Hospital, Valhalla, N. Y.

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TABLE I. Endpoints of Anti-Lansing Neutralizing Antibody in Serum of Monkeys During the Year Following the Last Intramuscular Vaccination Dose.

Mo. after vaccination						0	2	4	6	8	10	12	1	4	5
	1947						1948							1949	
	9/16	9/16,23	10/7,23	10/30	11/21	11/28	1/19	3/23	5/19	7/16	9/16	11/19	12/17	3/9	4/18
		↓↓	↓↓	i.c.	↓							↓			
C993	0			3.6	—	3.1	2.2	2.0	1.4* 1.5	1.0	1.4	1.5	3.4	2.4	2.5
C996	0			2.5	1.9	3.5	3.0	2.2	2.0	2.0	1.6	1.5	3.5	2.5	2.7
C998	0			2.4	2.2	2.8 3.3	3.5	2.9	2.7 2.7	1.8	2.5	2.3	3.0	1.7	2.4

↓ Monkeys received by intramusc. inj. 1 cc Lansing monkey spinal cord pools, as follows: On Sept. 16, 23, and Oct. 7, 23, 1947, rhesus C993 received a 5% suspension of pool IX(7); C996 and C998, a 2.5% suspension. On Nov. 21, all 3 monkeys received a 20% suspension of pool VII (C193). On Nov. 19, 1948, all 3 received 10% suspension of pool VII (C193).

i.c. On Oct. 30, 1947, all 3 monkeys were given intrathalamic challenge dose of 0.8 cc 10% suspension of pool IX(7). None showed signs of poliomyelitis.

* Duplicate figures indicate result of repetition of the intracerebral mouse neutralization test. Results reported as negative log of serum dilution at LD_{50} (before mixing with an equal volume of mouse brain virus suspension containing 20 LD_{50}).

were bled three more times in the following 5 months. The results of intracerebral mouse neutralization tests on these various sera will be reported and discussed here.

Materials and procedures. Three *M. mulatta* in good physical condition, weighing between 5 and 7 lb proved tuberculin negative to an intradermal injection of O.T. in the upper eyelid. The vaccination experience of these 3 individuals (C993, C996 and C998) in a series receiving graded vaccination doses has already been reported(3). The schedule of injections into the calf muscles (alternating sides) is given under Table I. The 50% paralyzing dose of Lansing pool IX(7) was 4.8 by intracerebral titration in monkeys; the PD_{50} of pool VII (C193) = 5.3 as reported earlier(3). Five months following the last vaccination dose (the end of the period shown in Fig. 1 and Table I), all received an intrathalamic challenge with 0.8 cc of 10% suspension of Lansing pool IX (D186). This pool has not been titrated, but was made under conditions which have regularly yielded pools with PD_{50} = 4.8 to 5.3. Two normal rhesus controls were injected simultaneously with the same suspension. Samples of heart's blood were taken from all 3 monkeys before vaccination, 6 weeks after beginning vaccination (just before intracerebral challenge) and again 3 and 4 weeks after intracerebral challenge. From the time of the last intramuscular vaccination dose (given 3 weeks after intracerebral challenge) blood samples were taken at two months' intervals for a year. After the intramuscular step-up dose at the end of that period, 1, 4 and 5 months' samples were obtained. During their residence of over a year and a half in the animal house, C993 and C996 remained in excellent physical condition. C998 developed diarrhea twice, but recovered on treatment with carbasone and sulfadiazine. Serum endpoints were measured by intracerebral mouse neutralization tests, according to the method used previously(7). Mixtures of 10-fold dilutions of serum in saline were made with equal volumes of a dilution of Lansing mouse CNS suspension (brain stem and spinal cord). The dilution of virus was such that the injecting dose of 0.03 cc contained 10 LD_{50}. Serum endpoints of 50% mortality of mice were calculated according to Reed and Muench(8).

Experimental results. Table I summarizes the experience of the three monkeys with Lansing virus and shows the endpoints found

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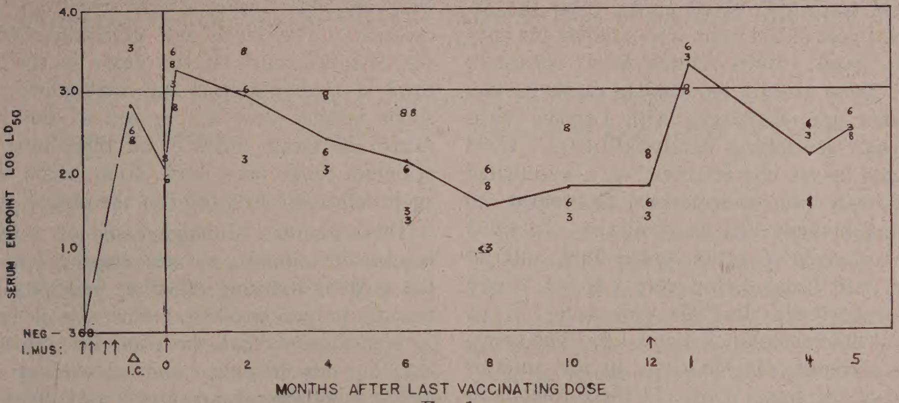

Fig. 1.

Duration of serum antibody in monkeys for the year following last intramuscular vaccination dose.

by serum neutralization tests. A few tests run in duplicate indicate differences in endpoints with a given serum of 0 to 0.5 log which is consistent with previous experience on reproducibility(7). The serum endpoints are represented graphically in Fig. 1. A line has been drawn through the mean values of the endpoints of the three sera taken at a given time. It can be seen that the individual endpoints of the 3 sera did not vary markedly from each other at any given time. A horizontal line has been drawn through the level of serum endpoint = 3.0, since it had been found in earlier experience(3) that serum endpoints in the range of 3.0 to 3.6 were optimum after such a course of vaccination. Such levels of serum antibody were associated with 100% immunity (in 37 monkeys) to the intracerebral challenge with virus. In the range of serum endpoints from 2.0 to 3.0 about half the individuals resisted. Although the 3 monkeys considered here received somewhat less than the optimum concentration of virus suspension required to insure the highest level of immunity, all three had survived intracerebral challenge and thus became available as resistant animals for study of duration of immunity.

An additional intramuscular vaccination dose of active virus 3 weeks after the intracerebral challenge boosted the serum endpoints in all three animals into the optimum range of 3.0 and over. Thereafter, as shown by serum neutralization tests done at 2 months' intervals, the serum levels fell gradually until 8 months. For the last 4 months of the year the endpoints remained at a level with a mean at about 1.8. Optimum serum endpoints were again attained in all 3 in response to an intramuscular booster dose at the end of the year. At 4 and 5 months after this booster dose, the endpoints again dropped to a moderate level.

To complete the history of these monkeys, beyond that shown in Fig. 1 and Table I, it should be added that at 5 months after the final booster dose, all 3 received an intracerebral challenge with active Lansing virus. Two normal rhesus monkeys, E854 and E855, inoculated at the same time showed paralysis on the 7th and 5th days after intracerebral injection. C993 and C996 showed no signs of poliomyelitis, but C998 developed paralysis beginning 7 days after challenge. This paralytic rate of one out of 3 vaccinated individuals with serum antibody levels ranging from 2.4 to 2.7 is consistent with earlier experience with a larger number of monkeys (3). With antibody levels in this intermediate range, about half of the group of monkeys became paralyzed following challenge. C993 and C998 were sacrificed one month after intracerebral challenge, whereas C996 died from unknown cause. From the 2 individuals sacrificed, samples of serum and spinal fluid, as well as suspensions of anterior horn of the spinal cord, and visual cortex from the occipital pole of the brain were studied for antibody levels. C993 showed levels consistent with those already reported in monkeys vaccinated and challenged with Lansing virus without developing paralysis(9,10). C998 showed levels characteristic of a vaccinated individual who nevertheless succumbed to typical poliomyelitis on challenge. In C993 the endpoints of serum, spinal fluid, anterior horn and visual cortex were 2.6, 0.5, 0 and 0.7 respectively; in C998 they were 3.2 (no spinal fluid sample), 4.2 and 1.8. The greatest difference was obviously in the anterior horn of the spinal cord. In the former individual which showed no poliomyelitis, there was no antibody found in this susceptible area, whereas in the latter, convalescing from poliomyelitis, highest levels were found in this area which had been infected, as consistent with earlier findings(9,10). The serum levels found at the time of sacrifice have not been included in the table since an intracerebral rather than intramuscular injection of virus intervened. In C993 the serum endpoint did not change after intracerebral challenge (2.5 before and 2.6 after challenge). This is similar to our previous findings in a total of 6 monkeys which had also been vaccinated with Lansing virus. Serum antibody levels were found to be the same before and after intracerebral challenge with Lansing virus. In this regard, the serum of C998, which rose from 2.4 to 3.2 is an exception for which there is no apparent explanation.

Discussion. Having achieved a method of effective immunization of monkeys with poliomyelitis viruses, it became possible to study the vital question of duration of such immunity. Monkeys vaccinated intramuscularly to the extent that their sera showed optimum levels retained demonstrable serum neutralizing antibody during a year free from vaccination. The levels fell gradually during the first two-thirds of the year; in the last third, a moderate level was maintained. A single booster dose at the end of the year raised the serum levels of all three into the optimum range once more, from which they again fell in the first third of the second year.

These findings, although based on a small number of animals, are encouraging towards the goal of inducing effective, enduring immunity to poliomyelitis. However it must be remembered that the procedure used in inducing this immunity was not without risk to the experimental animals(2) and therefore cannot be considered for human use. It should be added that monkeys have also been vaccinated with poliomyelitis viruses inactivated with formalin to the extent that no remaining active virus was demonstrable(11). In the monkeys vaccinated with inactive virus, antibody levels fell more rapidly than in the present series of monkeys vaccinated with active virus.

Summary. The level of serum neutralizing antibody to Lansing poliomyelitis virus has been followed in three rhesus monkeys. After a course of intramuscular vaccination with active virus, which rendered the animals resistant to intracerebral challenge with a large dose of virus, they received a final vaccinating dose intramuscularly. During the year following this final dose, no more vaccine was given. Their antibody levels, optimum at the start, fell gradually during the first 8 months, then remained at a moderate level for the last 4 months. At the end of the year, a booster dose of active virus given intramuscularly raised their antibody to optimum levels once more, from which they gradually drifted downward.

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Adaptation of Influenza B Virus to Mice by Use of Certain Adjuvants.* (18181)

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Variations in influenza viruses have been studied by a number of investigators(1). Hirst(2) found that strains of influenza A virus isolated in the embryonated egg multiplied readily in mouse lung, and reached a high titer. Early passages were unaccompanied by any visible lethal or pathogenic effects, although after 4 or 5 passages visible reactions occurred. He could not show such adaptation to the mouse with influenza B virus. Likewise, comparing influenza A and B strains from 3 epidemics, he found(3) B strains unstable on freezing and storing at low temperatures. Irregular and spotty virus titers, rapid adaptation to the amniotic sac, and failure to adapt to mice without prior ferret passage were additional characteristics. Sugg (4) studied an egg-adapted strain of influenza A prime virus from which mice died rapidly with complete pulmonary consolidation after inoculation of allantoic fluid. However, lung suspensions did not produce pulmonary lesions on passage. This virus was readily propagated in mice, developing a high degree of virulence on serial passage. He suggested that virus multiplication in the mouse lung was due to a variant of the egg strain.

In view of the fact that influenza is often accompanied by secondary bacterial infections, it was thought that the addition of bacterial cells or cell extracts to virus preparations of low virulence might enable the virus to increase in virulence and establish itself in the experimental animal. Results of such an adaptation procedure are reported here.

Materials and methods. The influenza virus used in this study was a B strain (No. IB45) which had been isolated in Iowa City during an epidemic in 1945(5). This virus possessed little or no virulence for mice, produced no initial pulmonary consolidation, and did not maintain itself on serial passage in these animals. Cultivation by inoculation into the amniotic and chorioallantoic sacs of embryonated eggs was possible, although titers were low and irregular, rarely reaching an agglutination titer of 1-1600 against 0.25% chicken red blood cells. Virus from the second egg passage was available as seed. A third egg passage was carried out with a 1-3 dilution of the above virus using 11-day embryonated eggs inoculated into both amniotic and chorioallantoic sacs. A stock supply of this third passage virus, undiluted or mixed with an equal volume of hormone broth, was stored on dry ice in rubber-stoppered glass vials. It was not believed that adaptation to the egg had progressed very far, and the virus had never been in mice. This virus was diluted 1-3 or 1-5 and inoculated into eggs at the start of all tests to obtain sufficient virus for mouse inoculation. This undiluted fourth passage virus in chorioallantoic fluid was mixed with the adjuvant in the proportion of one part adjuvant to 2 parts virus for inoculation.

Bacteria used as adjuvants included *Hemophilus influenzae* type b, freshly isolated at the beginning of the experiment, a Group A beta hemolytic streptococcus, a type III *Diplococcus pneumoniae,* and *Vibrio comma.* Organism suspensions were prepared in concentrations comparable to a No. 2 barium sulfate standard. In addition various nonbacterial preparations of particulate nature

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were used, including fresh and heated chicken red blood cells, starch, kaolin, and powdered glass. Several preparations of soluble material were also used, including glycine extracts of bacterial cells prepared by the method of Cowles(6) and Maculla and Cowles(7), filtrates of frozen and thawed cells, purified polysaccharide of group[†] III pneumococcus, laked chicken red blood cells, and gum arabic.

Four-week-old Swiss mice were used as test animals; 4-6 animals were used for each series. Inoculation was made by the intranasal route, under ether anesthesia, using 0.1 ml inocula. With each test, half of the mice were observed for signs of infection over a period of 10 days, and half were used for passage on the third or fourth day, at which time pulmonary lesions were noted. Ten per cent lung suspension (2 parts) plus adjuvant (1 part) were used for passage in mice. Tests for the presence of virus in the mice were made by inoculation of 10% mouse lung, with added penicillin and streptomycin (1), into the amniotic and chorioallantoic sacs of 11-12-day embryonated eggs. Two passages were made at 35°C. Virus was considered present when chicken red blood cells were agglutinated by the allantoic fluid of the test eggs. Periodic neutralization tests in mice and hemagglutination inhibition tests with chicken red blood cells checked the identity of the virus.

Results. The unadapted virus never maintained itself alone in mouse lung, and was always absent after the third passage in mice. On the other hand, the IB45 virus was able to propagate in mouse lung when certain bacterial suspensions were added to the inocula at each mouse passage. For example, when *Hemophilus influenzae,* type b, cells were added either living, heat-killed (57°C for 60 minutes), or streptomycin-inactivated, virus was present for 4 passages and indefinitely thereafter. After 8-10 passages in mice, pulmonary lesions were more extensive, and deaths occurred in shorter times than before, providing evidence that the virus steadily increased in infectivity as it became adapted to the mouse. Once the virus became established in the mouse, the added factor could be omitted without losing the virus.

Group A beta hemolytic streptococci, type III *Diplococcus pneumoniae,* and *Vibrio comma* organisms could replace *Hemophilus influenzae* as factors stimulating virus propagation in mice. In all instances, once the virus maintained itself for 3 or 4 passages, it remained present.

Three per cent chicken red blood cells, or red cells heated at 60°C for 30 minutes also aided propagation of the virus in mouse lung. Laked fowl cells had no effect. A glycine extract of *Hemophilus influenzae,* type b, cells aided the growth of influenza B virus in mouse lung. Dialysis did not remove the active factor from the glycine extract, and when glycine was added alone to the virus in the amount used for cell extractions, it was ineffective. A filtrate of a 10-day broth culture of *Vibrio comma* could replace cells or the glycine extract. Another soluble preparation with which virus propagated was a purified Group III polysaccharide of *Diplococcus pneumoniae* used in 1% solution. The results of these experiments are summarized in Table I.

Summary and conclusions. The addition of a variety of bacterial cell suspensions, cell extracts, or non-bacterial substances to influenza virus, strain IB45, preparations at each passage in mice apparently aided the initially non-virulent virus in such a manner that it could establish itself in the mouse lung and propagate through repeated mouse passage. This addition of adjuvant had to be repeated at each passage for 4 to 8 passages, after which extensive lesions and regular deaths occurred in mice. At this time the adjuvant could be omitted and the virus would continue to propagate. Single addition of adjuvant at the first passage was not sufficient for the virus to establish itself in the mouse, since the virus disappeared after 3 passages. Virus alone was never present after the third passage in mice. There

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[†] This was a mixture of several types of polysaccharides, labeled Group III.

TABLE I. Effect of Adjuvants on the Adaptation of IB45 Virus to Mice.

Adjuvant	No. of trials	No. of passages	Virus
None	5	3,3,4,4,6	Ab
H. influenza, living cells, A*	2	3,27	P
’’ heat-killed cells, A	5	6,7,8,8,10	P
’’ streptomycin treated, A	1	8	P
’’ glycine extract, A	3	6,10,15	P
’’ glycine extract dialyzed, A	2	8,23	P
’’ living cells added once	1	3	Ab
’’ filtrate of cells frozen and thawed 20 times, A	1	8	Ab
D. pneumoniae, type III, heat-killed cells, A	1	7	P
Group A beta hemolytic streptococci, heat-killed, A	1	7	P
V. comma, boiled 1 hr, A	1	4	P
Pneumococcus Group III polysaccharide, A	1	6	P
V. comma, filtrate of 10-day broth culture, A	1	6	P
3% chicken red blood cells, A	2	4,8	P
’’ ’’ ’’ ’’ ’’ heated, A	2	4,8	P
Laked 3% chicken red blood cells, A	1	9	Ab
Powdered pyrex glass, A	1	8	P
Soluble starch, A	1	8	P
Kaolin, A	1	8	P
Glycine, A	1	4	Ab
1% gum arabic, A	1	8	Ab

A* = Added each passage. Ab = Absent. P = Present.

In the series in which living cells of *H. influenzae* were added, and 27 passages in mice made, the adjuvant was omitted after 4, 8, 12 and 20 passages, and the virus remained present from 5 to 15 passages thereafter; further passages were not made. Occasional titrations in mice showed infectivity titers of at least 10^{-4} to 10^{-5}.

In the series in which a dialyzed glycine extract was the adjuvant, it was discontinued after the 20th passage, and the virus continued to propagate; the animals died and showed lesions to the same extent as before.

is no evidence that any one of the preparations possessed greater action than the others, although the addition of killed bacterial cells seemed to give somewhat more rapid adaptation of the virus in the mouse than cell extracts or non-cellular substances. An influenza B virus that was initially non-infective for mice became infective when certain adjuvants of bacterial or non-bacterial origin were added to each passage of virus. A procedure such as this may be useful in the isolation and adaptation of influenza viruses in mice.

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Protective Effect of Salt on Reduction of Viscosity of Sodium Thymonucleate Solutions by Heat. (18182)

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The high viscosity of aqueous solutions of sodium thymonucleate, which is characteristic of the polymerized, asymmetric molecular state of this material, is readily reduced by a variety of agents. Among these are added salts(1,2), heat(1), changes in pH(3), and ultraviolet(4) and X-irradiation(5-8). The nature of the action of these agents on the nucleate is as yet obscure. The present communication is concerned with the effect of two of these agents, namely salts and heat, applied simultaneously.

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* Special Research Fellow, National Cancer Institute; on leave from the University of Osaka, Japan.

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Methods and material. Several preparations of sodium thymonucleate obtained by the modified Hammarsten procedure(5) were employed. The relative viscosity of aqueous solutions of these materials was measured in Bingham and Jackson type viscometers at a constant external pressure of 6 cm H_2O (cf 2). Salt mixtures with nucleate were prepared by adding known volumes of salt of designated concentration to equal volumes of nucleate solution. The heating experiments were performed by immersing solutions in a carefully controlled hot bath.

Experimental. Thymonucleate solutions in water show a slow but definite decrease in viscosity on standing. When, however, such solutions are treated with sodium chloride to 0.5 M concentration, there is an immediate fall in viscosity to a value which is maintained quite constant over many days. This phenomenon suggested that added salt might protect the nucleate against the spontaneous diminution in its viscous property and perhaps even against induced falls in viscosity by such agents as heat.

Stock solutions of 1.0% nucleate were diluted by equal volumes of either water or NaCl solutions of various concentrations. The mixtures were then heated at 70° for 20 minutes, cooled rapidly to 30°, and their relative viscosities measured at this temperature. At concentrations of 0.5 M NaCl or higher, heating had apparently no effect on the viscosity of the nucleate solutions. The data are given in Table I.

Similar results to those in Table I were

TABLE I. Protective Action of Salt on Heated Solutions of Sodium Thymonucleate.*

Final conc. of NaCl, M	Relative viscosity† Before heating	Relative viscosity† After heating
Control (no salt)	15.6	3.5
2	8.9	8.9
1	8.8	8.7
0.5	7.9	7.8
0.1	6.5	5.4
0.01	7.4	2.7
0.001	12.2	3.4

* Final concentration of nucleate 0.5%.
† 6 cm H_2O external pressure.

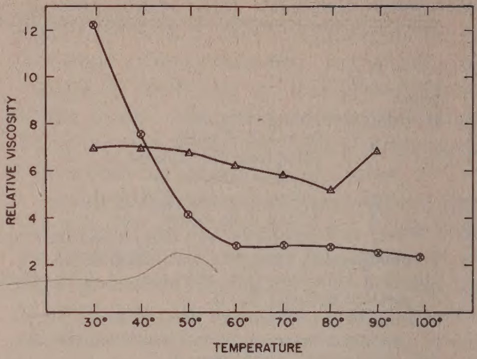

FIG. 1.
Effect of various temperatures on the reduction of relative viscosity, at 30° and 6 cm H_2O pressure, of sodium thymonucleate in the presence and absence of added sodium chloride. ○ 0.5% nucleate in water, △ in 1 M NaCl solution.

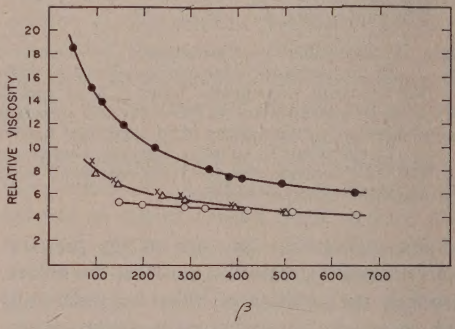

FIG. 2.
Relation of related viscosity at 30° of heated and unheated solutions of sodium thymonucleate in the presence and absence of added sodium chloride as a function of the velocity gradient β (cf. 2). ● 0.2% nucleate in water, unheated; ○ same solution after heating at 70° for 20 min.; ×, △ 0.2% nucleate in 1 M NaCl solution before and after heating at 70° for 20 min., respectively.

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TABLE II. Reversibility of the Protective Action of Sodium Chloride.

Mixture†	Relative viscosity* Before heating	After heating	After dialysis	After heating dialyzed sol.
Nucleate in H_2O	27.3	—	6.4	3.4
”	27.3	5	3.8‡	3.3
Nucleate in salt	12.8	—	10.2	3.9
”	12.8	12	10.2‡	3.6

* Viscosities measured at 30° and 6 cm H_2O external pressure.

† Final concentrations of nucleate and sodium chloride 0.5% and 1.0 M, respectively.

‡ Refers to the viscosity of the heated solutions after dialysis.

obtained with potassium chloride and magnesium chloride.

Fig. 1 describes the effect of heating at various temperatures on the viscosity of aqueous and of salt-containing mixtures with nucleate. Final concentrations of nucleate and of sodium chloride were 0.5 and 1 M, respectively. The period of heating was 20 minutes. At temperatures over 85°, mixtures of nucleate and salt sometimes yielded a small amount of white precipitate.

The structural character of the viscosity of the nucleate heated in the presence of salt is revealed in Fig. 2.

The reversibility of the salt protection was demonstrated by dialysis. Aqueous and salt-containing solutions of nucleate were heated, dialyzed, made up with water to equal concentrations of nucleate as measured spectrophotometrically in the ultraviolet, and heated again. Several such experiments revealed without exception that after loss of salt by dialysis the nucleate solution suffered a loss in viscosity on heating. A sample experiment is described in Table II. An interesting finding is that not only does salt protect against the effect of heat, but also apparently against the equally marked effect of dialysis on reducing the viscosity of the nucleate solution.

In another series of experiments, water and sodium chloride solutions (2M) were added to equal volumes of heated 1.0% nucleate solutions in water. Only a marked decrease in viscosity resulted after addition of the salt solution. The salt must be present while heating the nucleate solution in order to prevent the viscosity drop.

Summary. The viscosity of aqueous solutions of sodium thymonucleate is reduced both by heating and by addition of sodium chloride and other salts. If, however, the nucleate solution is heated in the presence of a sufficiently high concentration of salt, there is no further decrease in viscosity beyond that induced by the salt. The protective effect of the salt is reversible, for if the salt is removed by dialysis from the heated nucleate-salt mixture, the residual aqueous solution of nucleate again suffers a marked reduction in viscosity on heating.

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Human Plasma as a Source of Prothrombin in the Ac-Globulin Assay.*

(18183)

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The approach to the problems of blood coagulation from the point of view of studying single factors received a major stimulus with the development of the 2-stage prothrombin assay by Warner, Brinkhous and Smith (1). This provided for the measure of prothrombin, thrombin and antithrombin with a minimum of interference from other factors. Two decades later, Ware and Seegers(2) based their Ac-globulin assay on the 2-stage method and provided the investigator and clinician with another tool in the study of coagulation problems. By using relatively pure prothrombin, thromboplastin and fibrinogen and, by dilution, rendering inactive the various anticoagulation factors, they created readily reproducible Ac-globulin "curves." Because the preparation of prothrombin in relatively pure form is a difficult process requiring considerable experience, the assay of Ac-globulin has been limited to relatively few laboratories.

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* This study was supported by grants from Mr. Wm. H. Kidston, the Otho S. A. Sprague Memorial Institute Fund and the Arthur Dean Bevan Endowment (Anna L. Bevan Trust).

1. Warner, E. D., Brinkhous, K .M., and Smith, H. P., *Am. J. Physiol.,* 1936, v114, 667.

Owren's(3) treatise on the discovery and assay of Factor V (Ac-globulin) describes the use of oxalated human plasma as a source of prothrombin in the one-stage assay of the prothrombin accelerator. It is the purpose of this paper to describe the adaptation of this source of prothrombin to the 2-stage assay for Ac-globulin. Fahey, Ware and Seegers(4) have shown that both prothrombin and Ac-globulin are relatively stable in citrated, stored plasma, the former being more stable than Ac-globulin. In oxalated plasma, however, Ac-globulin disappears rapidly, being unmeasurable by the 2-stage assay in most stored human plasma samples in 10 to 15 days when kept at 4°C. The prothrombin during this time diminishes by 10 to 30% of its original measurable potency. Inasmuch as the required concentration of prothrombin in the last step of the 2-stage Ac-globulin assay is 1.67 Iowa units, this remaining quantity provides more than an adequate amount of prothrombin. It allows also for the dilution feature of the 2-stage test which is important in the neutralization of certain anticlotting factors.

Collection and processing of human plasma. A healthy human blood donor whose prothrombin level is at least 80% of normal is selected and his blood is drawn by clean puncture and aseptic technic into a flask containing 25 ml of 2.5% potassium oxalate. Ordinary blood bank equipment is satisfactory for this procedure. The flask is gently agitated during collection to insure uniform mixing of blood and oxalate. When the combined blood and oxalate measure 250 cc the needle is withdrawn. Proportionately larger amounts may be collected if desired. The blood is stored overnight at 4°C and the following day the plasma is siphoned off aseptically. It is then stored in a glass container stoppered with sterile gauze or cotton. The loss of Ac-globulin is apparently an oxidation process because its loss is materially retarded if the storage flask is hermetically sealed. The plasma is assayed at 3-day intervals for Ac-globulin activity. When it becomes unmeasurable by the 2-stage assay or when the one-stage prothrombin assay shows a timing of over 100 seconds (normal 11 to 12 seconds) the plasma is divided into accurately measured 0.5 ml samples and stored at –35°C until used. It will remain uniformly stable and reactive for several months.

The assay for Ac-globulin is carried out according to the method of Ware and Seegers (2). 0.5 ml of the prepared plasma is diluted in a graduated cylinder 16 to 20 times with saline. (The exact dilution required can be determined when the curves are later plotted. In estimating the dilution required it is helpful to know the prothrombin content of the stored plasma as measured by the 2-stage method. Inasmuch as the prothrombin contained in the solution added to the fibrinogen in the second stage of the assay should be 1.67 Iowa units, the dilution factor can be calculated when the prothrombin unitage is known.) 0.1 ml of the unknown plasma collected by a previously described technic (5) is added and thoroughly mixed with the diluted prothrombin. 1.0 ml of this is next added to 1.0 ml of a thromboplastin solution adjusted to proper activity. (0.1 ml of Soluplastin made by Schiefflin and Company, di-

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2. Ware, A. G. and Seegers, W. H., *J. Biol. Chem.*, 1948, v172, 699.

3. Owren, P. A., *Acta Med. Scand.*, 1947, Supp. v194, 1.

4. Fahey, J. L., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1948, v154, 122.

5. Fahey, J. L., Olwin, J. H., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 491.

luted to 15.0 ml has been satisfactory. The dilution will vary with different lots of the material.) 1.0 ml of the mixture of diluted prothrombin, unknown plasma and thromboplastin is added to 2.0 ml of a substrate containing 1 part of a 1% $CaCl_2$ solution, 1 part of imidazole (1.72 g in 90 ml of 0.1 N HCl, this solution then made to 100 ml with water), 2 parts of a 15% acacia solution (calcium-free) and 5 parts of saline. After 10 minutes, 0.4 ml samples of this final mixture are added at accurately timed intervals of 2 minutes to 0.1 ml samples of a 1% fibrinogen solution and the clotting times plotted as described by Ware and Seegers(2). By comparing the curve with those obtained from normal control plasmas the Ac-globulin content of the unknown plasma may be calculated in per cent of normal.

The use of human stored plasma as a source of prothrombin is subject to the same criticism that applies to any process which does not provide for the absolute control of all factors other than the one being measured. Assays in our laboratory for Ac-globulin in the whole stored plasma show less than 0.1% of the normal amount. Antithromboplastin is diminished and in some instances destroyed by freezing(6). Approximately 75% of the normal antithrombin remains, and less than 25% of the physiologic concentration of fibrinogen. Heparin is uncontrolled. All of the above factors including, of course, prothrombin are diluted 20 to 24 times in the final substrate. All of these considerations must be kept in mind but need not interfere with the utility of the method in most instances. A number of comparisons have been made with purified prothrombin[†] and the crude prothrombin substrate herein described. The Ac-globulin curves are readily reproducible and compare favorably with those obtained with the use of the purified material.

Summary. Oxalated human plasma deficient in Ac-globulin as a result of storage can be used as a source of prothrombin in the 2-stage assay of Ac-globulin.

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6. Tocantins, L. M., Personal Communication.

[†] Dr. Walter H. Seegers kindly supplied us with purified prothrombin.

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A Complement-Fixation Test for Poliomyelitis Virus. (18184)

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For several years this laboratory was concerned with a practical serological test for determination of infection with poliomyelitis virus, apart from that of neutralization. Conventional hemagglutination and precipitin tests failed, as did those by means of complement fixation (CF) which occasionally offered incomplete reactions not totally specific and not in any satisfactory way sufficient as a dependable, practical procedure(1). Hitherto Loring *et al.*(2) stated that CF could be observed, but a degree of non-specificity obscured certain results. Roberts(3) brought forward a flocculation test with a complex antigen; its value has not yet been established by other workers. During this investigation the MEF1 strain of poliomyelitis virus(4) was used, this strain being of the Lansing type but responding with a more uniform reaction and exhibiting a higher LD_{50} titer in mice than the Armstrong-Lansing strain (5).

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[*] Acknowledgement is made to Major Leslie C. Murphy, to Major Ralph O. Anslow, to Joan FitzGerald and to Henry Koch for their invaluable aid. Thanks are due to Dr. David Bodian, Johns Hopkins University, for his generous gift of monkey sera.

1. Casals, J., and Olitsky, P. K., in *Diagnosis of Viral and Rickettsial Infections,* edited by F. L. Horsfall, Jr., Columbia University Press, New York, 1949, Chapter 6, pp. 57-82.

2. Loring, H. S., Raffel, S., and Anderson, J. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 385.

3. Roberts, E. C., *Public Health Rep.,* 1949, v64, 212.

The belief arose through these studies on a CF test, that were the virus secured in higher concentration and, perhaps, in a different milieu than adult brain, a successful antigen might be achieved. This belief was apparently justified by the results here described, by which a specific CF test could be successfully and easily performed.

It was found that newborn W-Swiss mice, 1 to 4 days old, relatively resisted the virus after its intracerebral injection. With increasing age to about 21 days, they showed a gradually developing susceptibility to reach then the familiar complete susceptibility of adults. A report by Sabin(6) of similar findings with Lansing virus then appeared which could therefore be confirmed in practically all respects. However a step further was taken; an attempt was made to increase the pathogenicity of the MEFl virus for the relatively resistant infant mice by serial intracerebral passage which included "blind" passages from the 6th to the 9th. The experiment was successful and will be described in detail in another article. The result was that a virus was obtained which was highly active in newborn mice and was of a relatively higher LD_{50} titer than that yielded by adults.†

With this infant-mouse adapted strain of MEFl virus as antigen, the following sera have yielded positive results so far: (a) mouse, immunized with MEFl virus, (b) cotton rat, immunized with the Armstrong strain of Lansing virus, (c) monkey, convalescent after infection with MEFl strain and (d) monkey, immunized with MV strain.

Antigen. The source of material for the preparation of antigen was brain and cord tissue from newborn mice infected intracerebrally with MEFl virus. Serial passages of this virus in 3- and 4-day-old mice, carried out every 3 or 4 days, resulted in an adapted strain which is now in its 32nd passage. The characteristics of the adapted strain of virus were: (1) from 20 to 80%—average 50% of the mice inoculated, were paralyzed or dead by the 5th day, and 90 to 100% by the 10th day; (2) the LD_{50} titer—using 4-week-old mice for titration—was $10^{-4.6}$ in passage 15, $10^{-4.5}$ in passage 23, and $10^{-4.8}$ in passage 28. By comparison, the LD_{50} titer of the standard MEFl virus when propagated in 3- to 4-week-old mice had an average value in 18 titrations of $10^{-3.3}$ with a spread of $10^{-2.9}$ to $10^{-4.2}$.

Preparation of the antigen. 3- and 4-day-old W-Swiss mice were injected intracerebrally with 0.02 ml of a 10^{-1} dilution of virus obtained from the 21st to 24th and the 25th to 28th passages. Mice that were paralyzed on the 2nd to the 5th day following injection were sacrificed and the brain and cord removed. When enough material was available—from 15 to 20 g obtained from 75 to 100 mice—an antigen was prepared using essentially a method previously described (1,7), consisting of repeated extractions with acetone and ether. Following the last ether extraction, the dry material was resuspended in physiological saline solution in proportion of 1 part of diluent to 1 part of wet weight of CNS tissue. The supernatant obtained following centrifugation at 10,600 rpm for 1 hour constituted the antigen; merthiolate in final dilution of 1:10,000 was added and the

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4. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *Science,* 1943, v98, 452.

5. Yager, R. H., Olitsky, P. K., and Lahelle, O., *J. Bact.,* 1949, v58, 112.

6. Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 394; see also Dalldorf, G. in *Pathogenesis and Pathology of Viral Diseases,* edited by J. G. Kidd. Columbia University Press, New York, 1950, Chapter 4, pp. 31-46.

† The success of the adaptation of the MEFl strain to newborn mice led to a trial with other neurotropic viruses. Thus far B virus has been successfully adapted to such newborn animals, with the result that it possessed a uniform reaction; *Herpes simplex* virus was found after such passage to have an LD_{50} titer in excess of that obtained in adults—a report will follow. Other neurotropic viruses, including Brunhilde and Leon strains of poliomyelitis virus are being studied for possibilities of such adaptation.

7. Casals, J., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 339.

antigen was kept in a mechanical freezer at –20°C. The yield of antigen was approximately 2/3 ml per g of tissue. Antigens similar to the above have been prepared with normal uninfected newborn mice and with such mice infected with the viruses of Japanese (J.B.E.) and Western equine encephalitis (W.E.E.) which were used as controls.

Sera. Immune mouse serum was prepared on separate occasions by injection of infant-mouse adapted virus into adult mice. The latter, which were 2 months old when immunization was begun, were given 3 intraperitoneal injections of virus in dilution 10^{-1}; each injection of 0.5 ml was given at weekly intervals. These mice were bled 10 days after the last injection. Immune sera against W.E.E. and J.B.E. viruses were prepared in a similar manner, with the respective viruses being propagated in infant mice. Prior to the injection of active W.E.E. and J.B.E. viruses, the mice received 2 intraperitoneal doses of formalin-inactivated virus.

Cotton-rat immune serum was prepared over 1 year before the experiments on adaptation of the MEFl virus to infant mice were begun and was kept frozen at –20°C. The Armstrong strain of Lansing virus was used; the animals were immunized with cotton-rat propagated virus, injected once intramuscularly and 3 additional times intraperitoneally; pools of serum were obtained 3 weeks after the first injection and 2 months later. Normal cotton-rat sera were used as controls; in one series of these, 3 of 4 were found to be slightly anticomplementary.

Monkey sera. The sera from 2 convalescent monkeys infected with the MEFl strain were available. These sera had been received seven years ago and had been kept frozen. Also available were pools of sera from monkeys immunized with the Brunhilde, Leon, and MV strains—the MV being a Lansing type virus—which were kindly supplied by Dr. David Bodian. Normal monkey sera were used as controls.

Test. The CF test was performed as previously described(1,7) employing overnight incubation at 2-4°C and a total volume of 0.6 ml per tube. All necessary controls were

TABLE I. Showing a Complement-Fixation Test with Newborn Mouse Brain Antigen and Various Antisera with Controls.

	Antigens																												
	MEF1							J.B.E.							W.E.E.							Normal tissue					Saline		
Serum in dil. (reciprocal)	2	4	8	16	32	64	128	2	4	8	16	32	64	128	2	4	8	16	32	64	128	2	4	8	16	32	2	4	8
Monkey 1, MEF1	4	4	4	2	0																	0	0	0	0	0	0	0	0
Monkey 2, MEF1	4	4	2	0	0			±	0	0	0	0			2	0	0	0	0								±	0	0
Monkey, Brunhilde	0	0	0	0	0			0	0	0	0	0			0	0	0	0	0								0	0	0
Monkey, Leon	0	0	0	0	0			0	0	0	0	0			0	0	0	0	0								0	0	0
Monkey, MV	3	3	2	±	0			0	0	0	0	0			0	0	0	0	0								0	0	0
Monkey, normal	0	0	0	0	0			0	0	0	0	0			0	0	0	0	0								0	0	0
Cotton rat, Pool 1, Armstrong-Lansing	4	4	4	4	4																	0	0	0	0	0	0	0	0
Cotton rat, Pool 2, Armstrong-Lansing	4	4	4	4	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						0	0	0
Cotton rat, Pool, normal	4	4	0	0	0	0	0	4	3	0	0	0	0	0	3	1	0	0	0	0	0						3	1	0
Mouse, Pool 1, MEF1	4	4	4	4	4																	0	0	0	0	0	0	0	0
Mouse, Pool 2, MEF1	4	4	4	4	3-4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						0	0	0
Mouse, Pool, W.E.E.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	4	4						0	0	0
Mouse, Pool, J.B.E.	0	0	0	0	0	0	0	4	4	4	4	4	4	4	0	0	0	0	0	0	0						0	0	0
Mouse, Pool, normal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Numbers 0 to 4 represent absence of and degree of complement-fixation; J.B.E., Japanese B encephalitis; W.E.E., Western equine encephalitis.

TABLE II. Showing a "Box-titration" of Antigen in Graded Dilutions Tested with Serum in Graded Dilutions to Determine Titer of Antigen.

MEF1 antigen (dilutions)	Serum															
	Cotton rat, in dilutions (reciprocal)								Mouse, in dilutions (reciprocal)							
	2	4	8	16	32	64	128	256	2	4	8	16	32	64	128	256
Undiluted	4	4	4	4	3-4	3	±	0	4	4	4	4	4	3	±	0
½	3	3	3	2-3	2	2	2	±	2	1	1	1	1	±	0	0
¼	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
⅛	1	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normal brain, undil.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

included in each test. It is emphasized that all sera included in each test were tested against 1 or 2 control antigens. In addition, 2 different lots of MEFl antigen have been prepared and 2 different groups of mice were immunized. The test could be repeated 2 and 3 times with the different sets of materials. Table I shows the results of such tests.

With the exception of sera that were anticomplementary, the specificity of the reaction was manifested. Furthermore, in a test with the Brunhilde and Leon types of hyperimmune monkey serum, it appeared that the result was type specific, *i.e.*, MEFl antigen only fixed complement in the presence of the Lansing-type antibody. Additional tests with convalescent sera, to be reported, will concern the possibility of cross-reactions between the serological types.

A titration in which serial dilutions of serum were tested against serial dilutions of antigen is shown in Table II.

Table II indicates that the antigen as prepared has a low titer, that is, undiluted antigen reacted with mouse serum, and 1:2 dilution with cotton-rat serum. Incomplete reactions were observed in higher dilutions. Methods for increasing this titer are now being investigated.

Summary. The results of experiments here reported show that the MEFl, a Lansing type of poliomyelitis virus, can be adapted to infant mice in which the virus develops an LD_{50} titer in excess of that found, as a rule, in adult mice. In the medium of newborn mouse brain, and in such an increased titer, the virus now lends itself to the preparation of a suitable antigen for specific and reproducible complement fixation.

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Further Studies of the Beneficial Effect of Glutathione on X-irradiated Mice.* (18185)

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The beneficial effect of glutathione and cysteine on the course of radiation illness has been previously reported(1-3). Glutathione injected subcutaneously 5-30 minutes prior to irradiation improves the survival rate and decreases the weight loss in X-irradiated mice (1). Cysteine has a similar effect in rats and mice when administered either intravenously or orally before irradiation(2,3). In addition, Smith, *et al.*(4), have shown that the protective action of cysteine is related to the dosages of cysteine and X-radiation. The present report consists of a series of experiments in which glutathione-injected and non-treated mice were irradiated and compared with respect to X-ray dosage-mortality, weight change, and the effect of controlled trauma on the surviving irradiated animals.

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* The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department.

† Project NM 006 012.05.01.

1. Chapman, W. H., Sipe, C. R., Eltzholtz, D. C., Cronkite, E. P., and Chambers, F. W., Jr., *Radiology*, 1950, in press.

2. Patt, H. M., Tyree, E. B., Staube, R. L., and Smith, D. E., *Science*, 1949, v110, 213.

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Methods. A total of 2343 male, inbred, Swiss Albino mice was used in a series of 4 experiments. Mice were obtained at weaning from the Institute's stock colony, housed in individual cages, and irradiated when they weighed 20-25 g. The methods for housing and irradiating mice in individual cages have been reported(5). Irradiation consisted of a single total-body exposure to the radial beam of a 2 Mev. GE Industrial X-ray unit (6). The radiation factors were: 2000 KVP, 1.5 ma; no added filter; intensity of the radial beam: 15.0 (±0.15) r/minute in air at 2 meters; HVL 4.3 mm Pb. Lethal dose curves were determined simultaneously in each experiment by initially placing all animals of the experiment in the radial beam of the 2 Mev. unit, and then removing cages of animals as they received their prescribed dose of X-rays(5).

In the first 3 experiments the following doses of X-ray were employed: 550, 650, 750, 850, 950, and 1050 r (air). There were at least 36 mice for each dose of X-ray in each experiment, of which 12 or more received 1.6 mg glutathione per g of mouse in a single subcutaneous injection before irradiation, 12 received an injection of normal saline equal in volume to the glutathione injections, and 12 were non-treated. For each of these experiments there were also 60 non-irradiated controls, 20 of which received glutathione injections, 20 received saline injections, and 20 were non-treated. In the fourth experiment the following doses of X-ray were employed: 550, 650, 720, 775, 850, 950, 1050, and 1150 r. At each dose of X-ray, at least 24 mice received 4.0 mg glutathione per g of mouse in a single subcutaneous injection before irradiation; 12 mice received a corresponding volume of normal saline; 12 mice received no treatment. In addition, there were 96 non-irradiated controls; 24 received glutathione injections, 24 received saline injections, and 24 were non-treated.

A 10% solution of glutathione was used in each of the 4 experiments. Glutathione was dissolved in distilled water and adjusted to pH 6.5 with 10% sodium hydroxide. The control saline solution was adjusted to the same pH. All injections were given 5-30 minutes before irradiation in the backs of the animals, with tuberculin syringes and 22 gauge needles. Mortality rates were obtained during the 28-day post-irradiation observation period of each experiment. All mice were weighed individually each day. The change in weight of the survivors from each dose of X-ray was calculated daily and expressed in per cent of their pre-irradiation weight.

For the study of the influence of trauma, the following procedure was employed: On the 29th day of each experiment, all surviving animals were subjected to the same degree of trauma by use of a Noble-Collip drum(7), which was operated at 42.5 rpm for 8 minutes (3 mice per drum). The immediate and 24-hour mortalities were recorded. The 24-hour mortality data of an additional 891 male mice, similarly traumatized, are presented with the data from the survivors of these 4 experiments. These mice were in 3 groups: (a) 512 were non-treated and non-irradiated,

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4. Smith D. E., Patt, H. M., Tyree, E. B., and Staube, R. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 198.

5. Chapman, W. H., Cronkite, E. P., Chambers, F. W., Jr., and Morgan, J. E., Naval Med. Research Inst., Bethesda, Md., 1950, Project NM 006 012.05.03.

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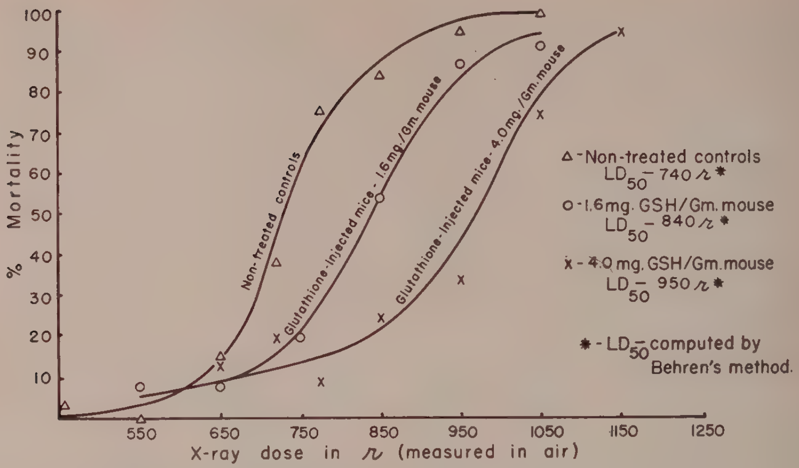

Figure 1:- Effect of Glutathione on mice exposed to 2 Mev x-ray radiation (single subcutaneous injection of Glutathione administered before irradiation.)

(b) 128 were non-treated and had survived 28 days following 800 r, and (c) 251 had received 1.6 mg of glutathione per g of mouse before irradiation and had survived 28 days following 800 r.

Results. Mortality data. The X-ray dosage-mortality curves for glutathione-injected and non-treated mice are presented in Fig. 1. The mortality data of all non-treated irradiated controls were combined, and the 28 day LD_{50}‡ for these mice was found to be 740 r. The mortality for the saline-injected control animals was not significantly different from that of the non-treated controls, consequently these data are not included in Fig. 1. The 28 day LD_{50} for the mice that received 1.6 mg glutathione per g of mouse was 840 r (experiments 1, 2, 3), and for the mice that received 4.0 mg glutathione per g of mouse, the 28 day LD_{50} was 950 r (Exp. 4). In these experiments 2.9% of 68 non-treated mice survived 950 r, while with 1.6 mg glutathione per g of mouse, 12.5% of 48 mice survived 950 r and with 4.0 mg glutathione per g of mouse, 65.6% of 30 mice survived 950 r.

It is of interest that with 4.0 mg glutathione

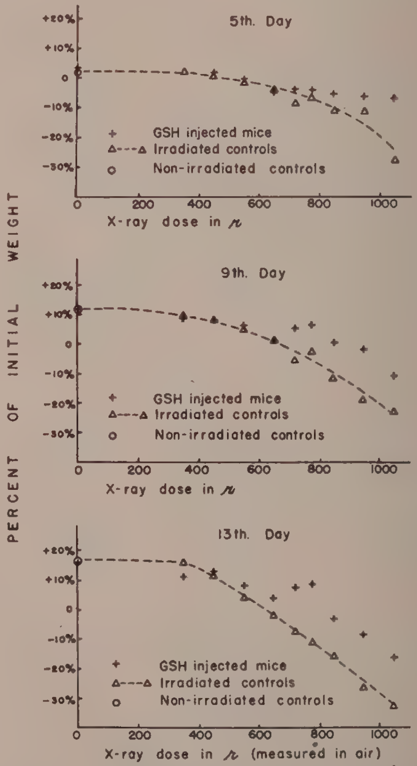

Figure 2.- Weight change of GSH-injected and non-treated mice surviving on 5th., 9th., and 13th. days, following single exposure to total body x-irradiation. (Pre-irradiation injection of Glutathione (4.0 mg./Gm. mouse)

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‡ Behren's Method(8)

8. Behrens, B. *Arch. f Exper. Path. w. Pharmakol.*, 1929, v140, 237.

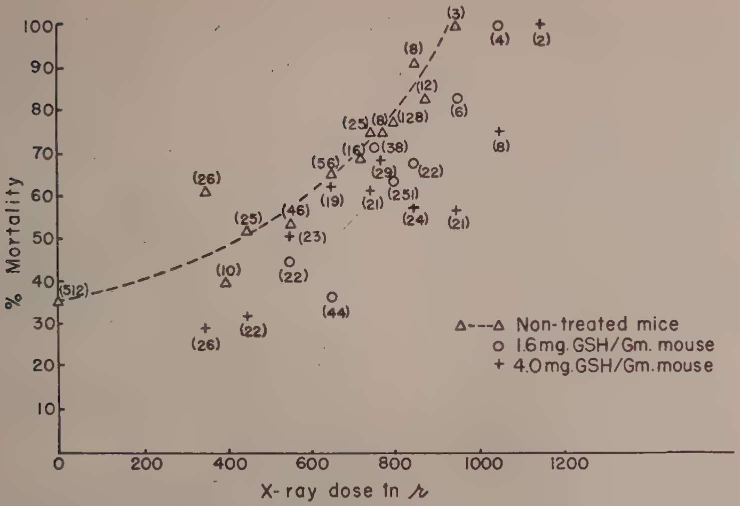

Figure 3.- 24 hour mortality produced by controlled trauma in GSH-injected and non-treated mice that survived 28 days following various doses of total body x-irradiation. The number of animals at each point is indicated in parentheses.

per g of mouse there were a few deaths within 3 hours after completion of the irradiation but, despite these early deaths, this larger dose of glutathione provided a greater over-all protection from radiation. It is also of interest that in the low lethal dose range (450-650 r), glutathione protection was not evident in these experiments. There were no deaths in the non-irradiated control animals of Exp. 1, 2, and 3. In Exp. 4, in the glutathione-injected non-irradiated control group, there was one death 24 hours after the glutathione injection.

Weight change. While weight change data were obtained daily for all animals, only those concerning Exp. 4 are presented in Fig. 2 as being representative of the entire group of experiments. The weight changes are expressed in per cent of the pre-irradiation weight of the surviving animals. It is apparent on the fifth day that with doses of X-ray above 800 r the glutathione-injected mice lost less weight; on the ninth day, the glutathione-injected mice with 720 r and above, lost less weight, and on the thirteenth day this difference in weight loss is evident in those mice with 550 r and above. At the time of maximum weight loss (13th day) there is, in the non-treated irradiated controls that received 450-1050 r, apparently a linear relationship between per cent weight change and X-ray dose.

Effect of trauma. The 24-hour mortalities of mice traumatized in the Noble-Collip drum are presented in Fig. 3. The mortality of 512 non-treated, non-irradiated mice was 36.5%, whereas, the mortalities of the non-treated, irradiated 29-day survivors varied from 40 to 100%, increasing with the X-ray dose. A curve is fitted to the 24-hour mortalities of these non-treated animals. The glutathione-injected survivors, in all cases, had a lower mortality than their non-treated controls.

Discussion. The results of these experiments confirm our preliminary observations on the beneficial effects of glutathione when this agent is injected subcutaneously in mice 5-30 minutes prior to X-irradiation(1). Patt, *et al.*(3) found glutathione effective in rats when injected intravenously before irradiation, but ineffective in mice and rats when administered orally. Two doses of glutathione were employed in the present experiments and, although the larger dose afforded more protection, the data are insufficient to quantitate the relationship between glutathione dosage and its protective action. Furthermore, the X-ray dosage-mortality curves show that the greatest degree of glutathione protection was evident at the higher dosages of X-radiation employed. Below 650 r there was no significant difference in the mortalities of the treated and non-treated animals. Either the numbers of animals used at these points were not sufficient to establish the true relationship or glutathione has less protective action in this range (450-650 r). If the latter is true, one might infer that different lethal mechanisms are induced by high and low doses of X-irradiation and glutathione is capable of modifying only those operating at the higher doses.

Change in body weight was followed daily in all animals as an indication of the degree of radiation injury sustained at the various X-ray dosages employed. The study of the effects of trauma on the twenty-eight-day survivors was utilized as an objective index of relative recovery from radiation injury. The results of these two studies, showing less weight loss and a decreased susceptibility to trauma in the glutathione-injected mice, together with the increased survival rates of the treated animals, indicates: (a) either recovery is accelerated in the mice that were injected with glutathione, or (b) less injury is sustained by the glutathione-treated animals per unit of radiation. The mechanism of this protective action of glutathione will be discussed in a subsequent report.

Summary. Glutathione injection prior to irradiation improves the survival rate, decreases the weight loss, and reduces the susceptibility to trauma of X-irradiated mice. Four mg glutathione per g of mouse is roughly twice as effective as 1.6 mg glutathione per g of mouse. In these experiments no protection was evident in the low lethal dose range (450-650 r).

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Relation of Vitamin A and "Lard Factor" to Disease Caused by Rancid Lard.* (18186)

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The biological effects of rancid fats, particularly of rancid lard, have attracted the attention of various examiners. Reviews of the literature were given by Burr(1,2) and by Quackenbush(3). All authors found that rancid lard causes a fatal disease in rats which has been surmised to be due to a combination of the oxidative destruction of known vitamins and unknown nutritional factors by the rancid lard and of a possible toxic effect of these fats.

In these studies, we shall try to demonstrate that the effects of rancid lard on rats can be prevented by vit. A or by the "lard factor" previously described by Kaunitz and Slanetz(4,5).

Methods. The examinations were carried

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* Aided by a grant from the Williams-Waterman Fund of the Research Corporation.

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4. Kaunitz, H. and Slanetz, C. A., *Fed. Proc.*, 1950, v9, 335.

out on a highly inbred colony of albino rats of the Sherman strain. Ten to 14 days after the birth of a litter, the mothers were transferred from "Rockland" rat diet to a vitamin A-free diet consisting of 10% fat, 30% alcohol extracted casein, 54% cerelose, 2% celluration, and 4% salt mixture. This diet was supplemented by 25 mg dl-alpha-tocopherol acetate and 10 mg free dl-alpha-tocopherol, 2 mg thiamine chloride, 4 mg riboflavin, 4 mg pyridoxine, 100 mg nicotinic acid, 1000 mg inositol, 1000 mg choline, 4 mg vitamin K, 300 mg p-amino-benzoic acid, 10 mg calcium pantothenate, and 100 IU Calciferol per kg of diet. In this diet, the fat used was the vitamin A-free residue of molecularly distilled lard (see below). After weaning at 21 to 25 days of age, paired litter mates were placed on the rancid lard diets, the compared groups having identical average weights. For the preparation of the vitamin A-free rancid lard diet, commercial lard was made rancid by aeration and heating at 90°C to a peroxide number of 256 and stored at a temperature of 4°C. Ten percent of this rancid lard replaced the lard residue in the above diet. Twice weekly, the rats received, in accordance with our previous practice(5), one drop of a suspension containing the various "B-factors". Fifty milligrams of ascorbic acid per cc were added to this suspension. As vitamin A supplements were used an aqueous solution of synthetic vitamin A palmitate stabilized by Tween containing 8000 units per cc[†] or a 2% solution of crystalline beta-carotene[‡] in linoleic acid or a sample of cod liver oil (Eimer and Amend) containing 900 units vitamin A per gram.

The "lard factor" was contained in the distillate fraction of freshly rendered lard which had been subjected to molecular distillation at 215°C and 10^{-3} mm Hg pressure. Seven per cent of the lard was removed by this procedure.[§] The distillate was carefully studied for vitamin A, both by the Carr-Price technic and by spectrophotometric methods[¶] and was found to be, for purposes of this experiment, practically free of any of the known forms of vitamin A. In growth studies, however, this distillate had exhibited considerable vitamin A activity(5). The residue of the lard left after molecular distillation was used as a source of vit. A-free fat. The experiments on food consumption and fecal fat excretion were carried out according to the usual practices with the modifications published before(5).

Experiments. When 12 female and 19 male weanling rats were placed on the vit. A-free rancid lard diet, they developed deficiency symptoms within 2 to 3 weeks, such as bloody mucous secretion from their nostrils, reddening of their eyes, and roughened, unclean fur. Death occurred among the emaciated animals 3 to 6 weeks after they had been placed on the diet, and there was no essential difference between males and females. The growth curves (Fig. 1 A,B,C) were recorded after Zucker and Zucker(6) by plotting the logarithm of the weight against the reciprocal value of the age; but, for facility in reading, they were marked off at convenient weights and ages. They indicated that no weight increase occurred after 2 to 3 weeks on the diet.

The detrimental effects of the rancid lard diet could be prevented by weekly doses of from 500-3000 units of vitamin A, administered either as the palmitate or as crystalline beta carotene or in cod liver oil; the growth curves (Fig. 1 A,B,C) were normal and all animals were without deficiency symptoms at

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[†] Dr. Leo A. Pirk of Hoffmann-La Roche, Inc., generously supplied us with vit. A palmitate and the other synthetic vitamins used. Dr. Augustus Gibson of Merck and Co. kindly gave us the vit. B_{12}.

[‡] Courtesy of the Barnett Laboratories.

[§] We are greatly indebted to Dr. Philip L. Harris and the other members of the Biochemistry Department of Distillation Products Industries, Division of Eastman Kodak, for supplying us with the molecularly distilled lard fractions and for vit. A analyses of the distillate.

[¶] Dr. J. J. Beaver of Columbia University kindly helped us with the spectrophotometric tests. Recent studies indicated that traces of carotene may be present in the lard distillate; the possible amounts are however insignificant when compared to the total vit. A activity of the material.

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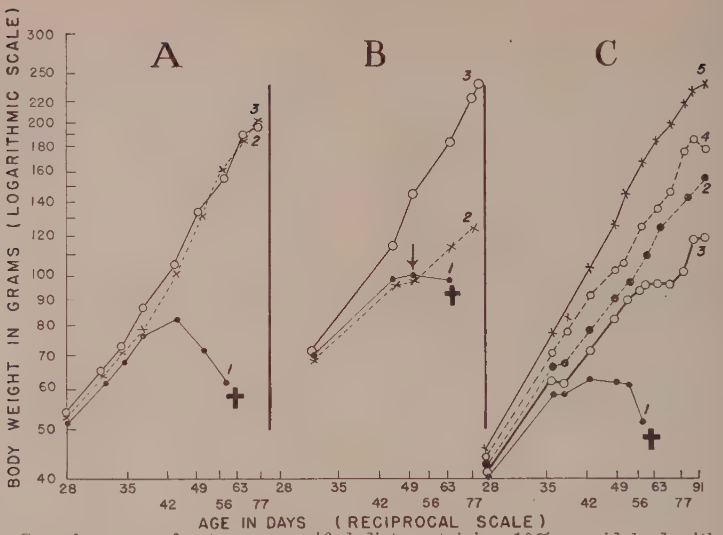

Growth curves of rats on a purified diet containing 10% rancid lard with and without various vit. A supplements.

Fig. 1.

(A) 1. Rancid lard diet (avg of 5 males). 2. Rancid lard diet plus 1000 units vit. A palmitate weekly (avg of 5 males). 3. Rancid lard diet plus 10 drops of cod liver oil weekly (avg of 5 males).

(B) 1. Rancid lard diet (avg of 8 males). 2. Rancid lard diet plus 1%, later (arrow) with 3% lard distillate (avg of 8 males). 3. Rancid lard diet plus 3000 units crystalline beta-carotene weekly (avg of 8 males).

(C) 1. Rancid lard diet plus 5% lard residue (avg of 9 females). 2. Rancid lard diet plus 5% lard distillate (avg of 6 females). 3. Rancid lard diet plus 5% lard residue plus 10 units of vit. A palmitate inj. weekly (avg of 8 males). 4. Rancid lard diet plus 5% lard distillate (avg of 6 males). 5. Rancid lard diet plus 5% lard residue plus 1000 units vit. A palmitate weekly (avg of 6 males).

12 to 14 weeks when the experiments were terminated. When 10 units of the vitamin A palmitate were injected weekly into 8 males (Fig. 1C), 6 were still alive at 100 days while all of the unsupplemented controls had died before they were 66 days old; but they soon developed deficiency signs, grew poorly, and stopped growing at 12 weeks. A single dose of 800 units vit. A palmitate fed at the age of 32 days to 3 male rats led to normal growth during the subsequent month. They survived their deficient litter mates but eventually became severely deficient.

In Fig. 1B and 1C are recorded the results of experiments in which 1-5% of the distillate fraction of molecularly distilled lard had been added to the diet. The growth of one group of females with 5% distillate (1C) seemed to be entirely normal; one group of males with 5% distillate showed no deficiency signs, but their growth was not as good as that of the group supplemented with massive doses of vitamin A. The "distillate" group did, however, much better than that receiving weekly injections of 10 units vitamin A. With 1% distillate (Fig. 1B), growth did not differ from that of the deficient controls; after the distillate was increased to 3%, growth was resumed, and these animals outlived their deficient paired litter mates.

In two groups of experiments, either 5000 units of crystalline beta-carotene or 50 units of vit. A palmitate were added to one kg of the diet. Weight curves and survival times did not differ from those of their litter mates on the unsupplemented diet, indicating that the vitamin A was rapidly inactivated. In contradistinction, at least part of the vit. A activity of the distillate was maintained after it had been added to the diet; it is improbable that this difference is merely due to the presence of an excess of the lard factor because, from previous work, it could be roughly estimated that one kilogram of the diet with 5% distillate contained about 200-500 units vitamin A activity. It is therefore possible to conclude that this factor is more resistant to the action (probably oxidation) of the rancid lard than ordinary vit. A.

It is probable that a considerable part of the lard factor was destroyed by the rancid fat because the addition of 5% to the rancid lard diet did not always give full protection, whereas 2% added to the A-free diet containing the residue fraction entirely prevented the deficiency symptoms.

In one experiment, growth and survival time of one group on the rancid lard diet (peroxide number, 256) was compared with a group on the same diet in which the fat source was the "residue" fraction (peroxide number, 30). The growth of the "residue" group was somewhat better for 2 weeks and they lived, on the average, for 46 days after the start of the experiment, while the rancid lard group died, on the average, after 39 days. Although the differences in growth and survival times were statistically significant, the vitamin A activity of the residue fraction must have been very slight because a control group on rancid lard receiving 3 units of vit. A palmitate weekly by injection did better than the residue group. Furthermore, the slight activity of the residue fraction could have been due to the fact that the lard residue may have contained traces of the "lard factor" not removed by the distillation. One is therefore tempted to conclude that differences in the peroxide number do not seem to be of great significance for the development of the disease.

Forty-five animals were used for the determination of the food consumption on either the deficient diet plus 5% lard residue or this diet plus massive doses of vit. A palmitate or the deficient diet supplemented by 5% lard distillate. The studies were done for one week when the rats were 43 to 50 days old. The intakes, calculated as the average consumption in grams per day per 100 cm² of body surface (Lee's formula) (7), were 3.4, 5.2 and 4.6 respectively. The lower food intake of the deficient group can obviously not be explained by aversion of the animals to the diet because the animals supplied with vit. A by extra feeding received the identical diet and consumed normal amounts; the low food consumption of the deficient group was, therefore, a consequence of the disease and not vice versa.

In 9 animals on the deficient diet, the fecal fat excretion was examined, and it was found that 97.5% of the ingested fat had been absorbed, indicating proper absorption of the rancid fat.

Discussion. From the above experiments, it is evident that the seemingly toxic effects ascribed to rancid lard can be prevented by extra feeding of large amounts of vitamin A. We are inclined to believe that many of the results obtained by previous examiners were due to the fact that insufficient amounts of vitamin A were fed—particularly if the vitamin A was included in the diet, where it must have been rapidly destroyed.

It seems, however, that the known forms of vit. A are, under natural conditions, frequently not the reason for the avoidance of "vit. A" deficiency because the more highly resistant substance or substances occurring in lard may be of greater importance than the easily oxidizable and not too widely distributed known forms of vit. A. This may be of particular significance when a substantial part of the diet consists of natural fats and oils which often contain very little of the chemically identified forms of vit. A.

Summary. 1. The fatal disease occurring in rats maintained on a purified, vit. A-free diet containing 10% rancid lard (peroxide No. 256) could be prevented by extra feeding of massive doses of synthetic vit. A palmitate or crystalline beta-carotene or cod liver oil. 2. Addition of the distillate fraction of molecularly distilled lard to the diet largely prevented the disease, although this fraction contains only traces of the known forms of vit. A. This "lard factor", when added to the diet, was more resistant to the action of rancid lard than beta-carotene and vit. A palmitate.

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3. Aversion to the diet or improper intestinal fat absorption were not responsible for the results. 4. It was concluded that rancid fats are not toxic *per se* and that, under natural conditions, the "lard factor" may play an important part in the prevention of the disease ascribed to vit. A deficiency.

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Progressive Decreased Glucose-tolerance and Glycogen-storage Following Acetoacetate Injection; Prevention by Insulin and Amellin. (18187)

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(Introduced by Arnold Lazarow)
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The hypothesis(1) that the gradual accumulation of intermediary fat metabolites such as beta-hydroxybutyric acid and acetoacetic acid might primarily be responsible for the progressive development of clinical diabetes and that these metabolites might exert a stimulating effect on the insulin secreting mechanism of the islet cells and ultimately lead to beta cell exhaustion through excessive strain, have been strengthened by recent publications from this laboratory(2,3, 4) and supported by Lazarow(5). He accounts for the increased fasting blood sugar levels, which are observed in men placed on a high fat diet(6,7) on the basis of this hypothesis. Houssay and Martinez(8) investigated the effect of different diets on the sensitivity of rats to alloxan and found that the toxic and diabetogenic effects were much more pronounced in the animals receiving a high fat diet. The harmful effects of a high fat diet were also observed by Opie(9), Dunham and Brunschwig(10), McKibbin *et al.*(11) and many others. It has recently been shown by Nath, Chakrabarti and Hatwalne(12) and Nath and Chakrabarti (13) that the injection of the sodium salt of beta-hydroxybutyric acid caused a marked depletion in the liver and muscle glycogen, thus suggesting that this compound may disturb phosphorylation.

The present paper shows that the injection of acetoacetate into rabbits reduces the glucose tolerance and the glycogen stored in liver and muscle. Amellin,* a plant product which was isolated by Nath(14) and shown to be highly beneficial in more than 50 clinical cases of diabetes(15-20), restored the glucose tolerance to normal and checked the depletion in liver and muscle glycogen. Comparative studies with insulin were also carried out.

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* This is a complex protein-like substance which contains a purine base, a thio sugar, and a phosphate group.

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Experimental. Twenty healthy rabbits, each weighing approximately 2 kg and having fasting blood sugar levels ranging between 106-116 mg per 100 cc, were selected. They were kept on a diet of germinated green gram *(Phaseolus mungo)* and green grass during the whole experimental period. The sodium salt of acetoacetic acid, which was prepared in the usual way, was given intraperitoneally. Since we previously(4) observed that the hyperglycemia induced by injecting the intermediary fat metabolites did not persist unless the successive dose was increased, the animals in our present experiment were given increasing doses of sodium acetoacetate, beginning with a daily dose of 50 mg/kg of body weight. The dose was gradually increased up to 300 mg/kg of body weight, as shown in the tables. The glucose tolerance test was carried out after fasting the animals for about 18 hours and the glucose was given subcutaneously in doses of 1.75 g/kg body weight. The method of Hagedorn and Jensen(21) was used for the estimation of the blood sugar. Some of the experimental animals were killed periodically and the glycogen stored in liver and leg muscle was determined according to the method of Good, Kramer and Somogyi(22). After 90 days, the experimental rabbits were divided into 3 groups. The animals in the first group were injected with sodium acetoacetate in increasing doses as mentioned before. The second group of animals was given 'Amellin' subcutaneously, 12 mg/kg body weight per day along with sodium acetoacetate as in Group 1, while the third group were given insulin subcutaneously, 0.5 unit/kg/day plus sodium acetoacetate. The comparative effects of these 2 drugs on glucose tolerance and glycogen storage were studied for an additional 40 days. After 130 days the dose of insulin was increased to 0.75 unit/kg body weight in some animals in order to see if this would facilitate glucose utilization.

Results. At the beginning of the experiment, prior to the injection of acetoacetate, the fasting blood sugar level ranged between 106 and 116 mg/100 cc. When a glucose tolerance test was performed the maximum blood sugar value was reached within a half hour and the blood sugar returned to the initial level within 2½ hours (Table I).

Following the administration of acetoacetate in doses of 50 mg/kg for 10 days, we found an indication of hypoglycemia in all the animals, as reported previously from this laboratory. After 30 days of injection, all the animals had passed over the hypoglycemic stage and showed definite indications of a fasting hyperglycemia. In most cases the blood sugar values during the glucose tolerance test did not return to the initial values within 2½ hours. After 50 days of acetoacetate injection on the shape of the glucose tolerance curve changed so that the maximum B. S. value was reached after 1½ hours and the B. S. values after 3½ hours are, in most cases, far greater than the initial ones. The curves were typically diabetic in character. By the 70th and 90th day the hyperglycemia increased further and there was a progressive decrease in glucose tolerance. At 90 days some of the initial B. S. values exceeded 190 and the average percentage increase of B.S. during the glucose tolerance test increased to 68% (normal = 35%). Average glucose tolerance curves at different periods of injection are represented in Table I. Tidwell and Axelrod(23), studied the effect of acetoacetate in rats for a period of only 4 weeks. During this time they observed hypoglycemia, but they did not observe the second hyperglycemic phase. This may be due to their failure to continue

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TABLE I. Average Glucose Tolerance of Rabbits Injected with Sodium Acetoacetate (Glucose Injected SQ. in Doses 1.75 g/kg Body Weight).

Days of acetoacetate* inj.	No. of rabbits used for glucose tolerance	Blood sugar in mg/100 cc blood										Avg % increase in blood sugar during glucose tolerance
		Initial		½ hr		1½ hr		2½ hr		3½ hr		
		Avg	σ	Avg	σ	Avg	σ	Avg	σ	Avg	σ	
0	20	112	2.7	229	21.1	153	13.9	112	4.0	113	2.5	35.2
10	19†	91	6.6	193	12.2	175	8.5	89	7.5	92	7.5	51.3
30	18	141	9.0	250	18.0	217	21.6	178	25.6	149	13.7	40.6
50	17	156	13.8	246	17.3	302	15.3	222	24.0	204	10.1	56.6
70	13†	165	18.2	261	11.8	335	26.9	255	20.8	234	25.6	64.8
90	10	171	20.7	283	22.3	347	26.2	274	28.8	250	24.9	68.3

* Daily dose of acetoacetate inj.: 1st to 10th day, 50 mg/kg; 11th to 30th day, 100 mg/kg; 31st to 50th day, 150 mg/kg; 51st to 70th day, 225 mg/kg; 71st to 90th day, 250 mg/kg; 91st to 110th day, 275 mg/kg; 111th day to 130th day, 300 mg/kg; (131st day onwards increase by 25 mg/kg every 20 days).

σ = Stand. dev.

† One rabbit died before the 10th day and 4 died between the 50th and 70th day. The other animals were killed for glycogen studies.

the study past 30 days or to a species difference in the response to acetoacetate. Nath *et al.*(24) have shown that acetoacetate can stimulate the biosynthesis of vitamin C in rats far more than in rabbits. As vitamin C is known to be highly beneficial in improving glucose metabolism(25) it might be expected that the hyperglycemic phase in rabbits would be more pronounced than is the case in rats.

Comparative effect of Amellin and insulin on acetoacetate induced diabetes. After 90 days when all the animals showed definite hyperglycemia and decreased glucose tolerance, the effects of insulin and Amellin were studied. Amellin was injected into 6 fasting animals in varying doses (20 mg, 30 mg and 50 mg/kg body weight) and its effect on the blood sugar was determined during the subsequent 3½ hours. The animals were fed for 4 hours and then allowed to fast for 16 hours. The same experiment was repeated on the 2nd day and the fasting 48 hour blood sugar level was also determined. Parallel studies with insulin (0.5 unit per kg) were carried out in four other animals treated in the same way. The results are shown in Table II. Six of these animals were given Amellin (10 mg/kg body weight/day) and 4 rabbits were injected with insulin (0.5 unit/kg/day) for 20 days; the injection of acetoacetate was continued in the doses stated previously. At the end of this period a glucose tolerance was performed. After an additional 20 days of combined acetoacetate plus Amellin or insulin injections the glucose tolerance was repeated. At this time the insulin dose was increased from 0.5 unit to 0.75 unit/kg and 3 rabbits received this increased insulin dose for 7 days after which time a glucose tolerance test was again performed. The results are shown in Table III.

It will be noted that Amellin treatment not only lowered the fasting blood sugar value but it also improved the glucose tolerance. Although insulin lowered the blood sugar temporarily it did not produce a permanent decrease in the fasting blood sugar level as did Amellin, for in the 3 rabbits that had received insulin for 47 days the initial blood sugar values ranged between 198 and 210 mg/100 cc 24 hours after the injection of insulin plus glucose.

Depletion in the glycogen storage and its restoration. The animals were killed periodically, *i.e.* after 10, 30, 90 and 130 days and the glycogen contents of the liver and muscle were determined in the usual way. The following table indicates the enormous loss of glycogen, as compared to normal animals. These data are in agreement with those observed in rats under similar condi-

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TABLE II. Immediate Effect of Amellin and Insulin on B.S. Values of Animals with Hyperglycemia Induced by Acetoacetate Injection for 90 Days. (Dose on the 90th day 250 mg/kg).

No. rabbits	Treatment		Blood sugar (avg values) mg/100 cc blood										
	Substance	Dose inj. (mg/kg)	Initial (fasting)	First day After				Initial (fasting)	Second day After				3rd day initial (fasting)
				½ hr	1½ hr	2½ hr	3½ hr		½ hr	1½ hr	2½ hr	3½ hr	
2	Amellin	20	154 ± 18	148 ± 17	175 ± 12	126 ± 6	115 ± 5	161 ± 7	119 ± 0.5	114 ± 1	110 ± 2	107 ± 1	126 ± 7
2		30	187 ± 1	181 ± 0.5	187 ± 1	206 ± 2	157 ± 3	184 ± 2	134 ± 1	132 ± 3	125 ± 3	122 ± 3	115 ± 3
2		50	205 ± 9	198 ± 2	165 ± 0	138 ± 2	101 ± 11	158 ± 32	155 ± 43	117 ± 9	109 ± 5	104 ± 7	123 ± 7
4	Insulin	.5 U	186 ± 7	126 ± 6	145 ± 6	157 ± 8	157 ± 15	176 ± 16	116 ± 9	139 ± 13	146 ± 5	164 ± 6	178 ± 13

TABLE III. Effect of Chronic Amellin and Insulin Treatment on Hyperglycemia and Abnormal Glucose Tolerance Induced by Acetoacetate* Injections.

Days of acetoacetate inj.	No. of rabbits	Treatment			Blood sugar mg/100 cc blood										Avg % increase in blood sugar during glucose tolerance
		Substance	Dose per day/kg	Days of treatment	Initial		½ hr		1½ hr		2½ hr		3½ hr		
					Avg	σ	Avg	σ	Avg	σ	Avg	σ	Avg	σ	
90	10	0	0	0	171	21	283	22	347	26	274	29	250	24	68.3
110	2	0	0	0	168	7	195	9	317	7	274	3	266	2	56.5
110	6	Amellin	10 mg	20	132	18	204	53	152	27	132	34	102	29	12.1
110	4	Insulin	.5 U	20	114	21	170	18	109	4	97	8	108	13	6.1
130	2	0	0	0	174	3	195	0	389	5	290	5	283	2	66.1
130	5	Amellin	10 mg + 12 mg	20 + 20	105	9	173	26	112	4	107	6	108	3	19.0
130	3	Insulin	.5 U	40	182	33	264	13	235	29	162	76	157	76	12.6
137	3	,,	.5 +.75	40 +7	213	13	280	9	313	9	122	14	139	19	0.5

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* For dose of acetoacetate see Table I.

TABLE IV. Glycogen Content of Liver and Muscle Taken from Rabbits Injected with Acetoacetate and Treated with Amellin (10-12 mg/kg/day) or Insulin (.5-75 U/kg).

No. of rabbits	Days of acetoacetate* inj.	Treatment	Avg body wt, g	Glycogen mg/100 g tissue	
				Liver	Leg muscle
2	0	0	1400	234.3 ± 12.4	138.8 ± 11.3
1	10	0	1620	175.2	60.5
1	30	0	1550	145.7	43.4
1	90	0	1100	86.0	37.5
2	130	0	2000	82.0 ± 5.2	37.0 ± 4
2	150-151	Amellin†	1295	230 ± 20	57.8 ± 25
2	150-151	Insulin†	1470	154 ± 14	48.5 ± 20

* Dose as in Table I.

† Administered from the 91st to the 150th day.

tions(12,13). The effects of Amellin and insulin on the restoration of liver and muscle glycogen are shown in Table IV. It will be noted that the repeated administration of Amellin to rabbits receiving acetoacetate restored the liver glycogen almost completely and the muscle glycogen to a lesser extent. Insulin was less effective in restoring liver and muscle glycogen than was Amellin. Although all the animals treated with acetoacetate became hyperglycemic and showed typical diabetic glucose tolerance curves and glycogen depletion, none showed glycosuria at the end of the 130 day experimental period. Injections were therefore continued in some animals to see if they could be made glycosuric on prolonged treatment. After 140 days of injection, glucose was injected for a tolerance test (1.75 m/kg) and one rabbit showed glycosuria (0.4% glucose in the 24 hour sample). The treatment with acetoacetate was continued and on the 160th day the same rabbit developed a spontaneous mild glycosuria (0.1% sugar in 24 hour sample). The animals which were killed periodically for the estimation of glycogen, were also studied morphologically. Tumors were found in the livers of all animals receiving only acetoacetate; their size varied proportionately with the period of injection. The kidneys also showed signs of progressive damage. No tumors were seen in the animals treated simultaneously with acetoacetate plus either Amellin or insulin for about 40 days. The kidneys of these animals were also found to be more or less normal.

It has been observed by Joslin(26) that human diabetes is frequently preceded by a period of obesity. Foglia(27) found that after the removal of 80 to 95% of the pancreas in rats there is an initial period of obesity with no manifest signs of diabetes. This stage is then followed by a period of alimentary hyperglycemia which, in turn, is followed by fasting hyperglycemia and glycosuria and finally a period of ketosis and death. This progression of diabetes in subtotal pancreatectomized rats parallels in many ways the development of human diabetes(27). As similar stages have also been observed in the experimental animals reported here, it is possible that this progression of diabetes induced by intermediary fat metabolites may represent an experimental diabetes which closely parallels the development of human diabetes.

The hyperglycemic effect of a high fat and low carbohydrate diet as observed by Himsworth(28) can also be accounted for in the light of these findings.

The beneficial effects of Amellin in animals with diabetes induced by ketone body injection might be due to either its ketolytic effect as observed by Nath and Habibul Islam (29) or to its action on enzyme systems. Amellin, which has been found to contain a thio group, may also exert its protective

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26. Joslin, E. P., *New Eng. Jour. Med.*, 1933, v209, 519.

27. Foglia, V. G., *Rev. Soc. Argent. de Biol.*, 1944, v20, 21.

28. Himsworth, H. P., *Clin. Sc.*, 1935-36, v2, 117.

29. Nath, M. C., and Habibul Islam, A. H. M., *Ind. Jour. Med. Res.*, 1948, v36, 61.

influence like glutathione, which in large doses has been found by Lazarow(30,31) to completely protect rats against the development of alloxan diabetes.

Summary. 1. The repeated daily injection of sodium acetoacetate in progressively increasing doses (beginning at 50 mg/kg body weight) caused a progressive hyperglycemia in rabbits after an initial hypoglycemic stage. 2. The glucose tolerance of these animals was decreased after 30 days of injection and the tolerance curve taken at 30, 50, 70, 90, 110 and 130 days became progressively more diabetic. 3. The shape of the tolerance curves changed considerably on prolonged treatment with acetoacetate and in most cases the blood sugar levels at 3½ hours were far greater than the pre-injection level. 4. The glycogen content of liver and muscle is gradually depleted, so that after 130 days of injection it was decreased 64.9% and 73.4% respectively. 5. When Amellin was injected daily for 40 days in doses of 10-12 mg/kg into these hyperglycemia animals it reduced the blood sugar to 105 mg/100 cc (normal value). Insulin, although it relieved the fasting hyperglycemia initially, had little effect on the fasting hyperglycemia in later stages. Increasing the insulin dose leads to death. 6. The glucose tolerance of the animals treated with sodium acetoacetate became absolutely normal after Amellin had been administered for 40 days, whereas the tolerance of similar animals treated with insulin was not normal at 40 days.

Addendum. After this paper was presented for publication Griffiths(32) reported that uric acid produced diabetes in rabbits only when their blood glutathione was lowered to about half the normal value. Acetoacetate injection has recently been found in this laboratory to lower blood glutathione considerably(33). These observations may help to explain how acetoacetate favors the development of hyperglycemia and how Amellin acts as a protective substance.

The authors are deeply indebted to Dr. Lazarow of Western Reserve University for his keen interest in this work.

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30. Lazarow, A., *Anat. Rec.*, 1945, v91, 24.

31. Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 441.

32. Griffiths, M., *J. Biol. Chem.*, 1950, v184, 289.

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Alpha Tocopherol in Treatment of Alloxan Diabetes in Dogs.* (18188)

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Reports have appeared in the literature on the use of alpha tocopherol, in treatment of diabetes(1,2). Recent publications reviewing therapy of diabetes mellitus have invariably made a plea for fundamental controlled work to be done on the use of alpha tocopherol in the treatment of diabetes mellitus(3). Therefore, it was considered worth while to study the effect of alpha tocopherol on alloxan diabetes in the dog.

Method of study. Ten dogs were rendered diabetic by a freshly prepared 5% solution of alloxan administered in intravenous doses of 65 to 75 mg per kilo body weight. The dogs were then divided into 2 groups, 7 comprising the experimental group, 3 the control group. The experimental dogs were treated with diet, insulin and alpha tocopherol. The control

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* These studies were supported by a grant from The Warren-Teed Products Co., Columbus.

1. Vogelsang, Arthur, *Medical Record*, June-July 1948, p. 1.

2. Butturini, Ugo, *Gior. di Clin. med.* 1945, v26, 90.

3. Joslin, E. P., *J.A.M.A.*, 1949, v140, 581.

TABLE I. Influence of Alpha Tocopherol on Insulin Requirements of Alloxanized Dogs.

Dog No.	Wt, kg	Fasting blood sugar, mg %	Alloxan dosage, mg/kg	Blood sugar after alloxan, mg %	Prot. zinc insulin daily units before α-tocoph.	α-tocoph. oral daily I.U.	α-tocoph. intramusc. 2-day intervals, mg	No. of days	Prot. zinc insulin concluding daily units
1	11.4	55	75	252	10	300	0	40	10
6	15.5	64	100	319	25	300-600	0	40	25
9	13.6	68	65	92	8	300-600	0	40	8
12	6.4	64	75	210	5	300-600	0	35	5
24	10.5	55	65	216	10	300	0	27	10
15	21.0	68	75	207	25	0	50-200	28	25
18	15.9	53	65	136	10	0	50-200	26	10
16 Control	17.7	57	65	104	5	0	0	36	8
17 Control	11.8	49	65	118	6	0	0	35	8
23 Control	14.5	55	65	74	5	0	0	33	5

dogs were treated with diet and insulin. In all instances protamine zinc insulin was used to control the diabetes. Blood sugar determinations were done before alloxan and approximately 48 hours after alloxan. Thereafter, blood sugars were determined at various intervals in order to follow the hyperglycemic state. Insulin dosage was established by daily morning urinalysis. After control of the glycosuria was definitely established, alpha tocopherol was administered to the experimental dogs an average of 4½ weeks. Five of the experimental dogs received oral alpha tocopherol daily, 2 of the experimental dogs received injectable alpha tocopherol every other day.

Results. Table I presents data on each dog. In all cases regulation of the diabetes was readily attained. The general nutritional status of all dogs was well maintained throughout the experiment. In no instance was the insulin requirement reduced in the experimental animals.

Summary and conclusions. 1. Alpha tocopherol does not alter the insulin requirements of alloxan diabetes in the dog.

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Hyperpigmentation in Acute Leukemia Treated with Folic Acid Antagonists.* (18189)

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The treatment of acute leukemia with folic acid antagonists has resulted in encouraging clinical responses. While it is recognized that such antagonists as Aminopterin and A-methopterin (Lederle) do not cure acute leukemia they nevertheless depress abnormal bone marrow response by correcting the dysplastic process and thereby prolong life in many instances. Folic acid antagonists can therefore be useful tools in studies designed to elucidate the abnormal cellular metabolism of the leukemic marrow. During our observations on 10 children who received the antagonists over long periods we were impressed by the development of hyperpigmentation in 7 patients. While the examination of tissues from all the patients is in progress, complete histologic data in one case is so striking that we wish to present this case history documented by microscopic evidence of marked epidermal pigmentation. Tissues from 4 other patients have provided preliminary evidence of similar findings. While it is true that Aminopterin prolongs life, this degree of pigmentation has not been noticed previously in cases of acute leukemia not treated with folic acid antagonists.

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* This investigation was supported in part by a research grant from the National Cancer Institute, U. S. Public Health Service.

A 10-year-old boy, admitted for treatment of acute leukemia received either 0.5 mg Aminopterin or 5.0 mg A-methopterin (Lederle) for six months. The patient gradually developed hyperpigmentation; first, at the ankles and wrists. This was followed by darkening of the skin at the nail folds of the fingers. The skin of the abdomen and back likewise became darker during the last 3 months of life. At the time of autopsy, approximately 9 months after the onset of the disease, the entire skin appeared appreciably darker than normal. Skin specimens from the ankle, wrist, abdomen and back were secured in order to ascertain the histological nature of the hyperpigmentation. Fresh autopsy specimens were studied by means of the dopa-incubation method of Becker(1). Other specimens of the same skin areas were fixed in 10% formalin and stained according to Mason's ammoniated silver nitrate technic(2) for the detection of dendritic melanoblasts and of any epidermal cells containing either premelanin or melanin granules. In view of the possibility that the darkening of the skin might have been due to abnormal deposits of iron-salts (hemosiderin) following transitory periods of icterus, ferrocyanide reactions according to the method of Perls(3) were made on some sections of the various skin specimens. These Prussian-Blue stains were negative, thus eliminating iron as a factor in the hyperpigmentation.

The dopa-reactions and ammoniated silver nitrate impregnations as well as unstained sections of the various skin areas revealed a true process of melanogenesis within the epidermis. Typical pigmentary dendritic cells (melanoblasts) were easily recognized. These cells gave a slightly positive dopa-reaction which is characteristic for the true melanin-producing cells and reveals the presence of an active oxidizing enzyme. Both the dopa and silver preparations brought into evidence the dendritic processes of these cells which pervade the intercellular spaces of the deeper layers of the epidermis. They contained premelanin and melanin and the process of a secondary transfer of melanin from the dendritic cells to ordinary epithelial cells was clearly under way. Additional melanin-containing cells were conspicuous, in fairly large numbers, in the upper dermis (papillary layer). These cells were identified as large phagocytic leucocytes which had merely ingested melanin granules produced elsewhere. Their melanin content consisted of coarse, irregularly sized granules, in contradistinction to the finely granular melanin within the melanoblasts. These phagocytes of the dermis were merely carriers (melanophores) and it can be presumed that they obtained melanin granules from the sites of abnormal melanin production within the epidermis, by way of the slowly seeping intercellular fluid from that layer of the skin. They are not concerned with the true process of melanogenesis which occurred within the epidermis. Such phagocytic melanin-carriers remain dopa-negative and when accumulated in large numbers may indirectly affect the color of the skin which would then have a slate-bluish tinge. The skin specimens of our cases were various shades of brown. It is well established that the elaboration of melanin is dependent on the enzymatic oxidation of tyrosine. The presence of dihydroxyphenylalanine (dopa) catalyzes the reaction and evidence is accumulating that the human melanoblasts contain tyrosinases capable of oxidizing tyrosine and related substances. Since tyrosinase is a copper-protein complex,

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3. Perls, M., Ferrocyanide Reaction in Lillie, R. D. *Histopathologic Technique* p. 124, The Blakiston Co.

several interesting relationships exist, especially those concerned with inhibition of the enzyme. It has been suggested by Lerner and Fitzpatrick(4) that sulfhydryl groups of glutathione and cysteine inhibit tyrosinase by binding the copper. It has also been postulated that in addition to the sulfhydryl compounds, the high oxidation potential of other substances also influences the copper containing enzymes so that they are unable to function optimally.

This paper does not provide data nor is any information available on the effect of folic acid antagonists on tyrosinase or on tyrosine metabolism, but several authors have pointed out the relationship of both folic acid and ascorbic acid to increased excretion of "tyrosyl" compounds. Woodruff and Darby (5) have shown that the increased excretion of "tyrosyl" compounds in scorbutic guinea pigs could be corrected either with ascorbic acid or folic acid. Evidence already exists that folic acid is concerned with tyrosine metabolism since Govan and Gordon(6) showed earlier that the premature infant's inability to cope with a high protein diet resulted in increased excretion of "tyrosyl" compounds which could be corrected by folic acid therapy or by ascorbic acid administration(7) and Johnson and Dana(8) have shown that ascorbic acid given to folic acid deficient rats can modify the loss of weight after the deficiency is induced by a diet containing sulfasuxidine. The chemical relationship of tyrosine to adrenochrome compounds also permits speculation regarding the role of the adrenal glands in the pigmentation of our patient. Since tests for adrenocortical function were all within normal limits, the possibility of an Addisonian type of pigmentation is unlikely in our patient.

Summary. Hyperpigmentation of the skin has been observed in several cases of acute leukemia receiving folic acid antagonists and histological studies in one patient confirmed the impression that true melanogenesis had occurred. The role of antagonists in influencing the tyrosinases concerned in melanin formation is unknown.

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Role of Detergent Complexes in Experimental Gastric and Duodenal Ulcers.* (18190)

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Dogs injected with histamine by the method of Code and Wangensteen(1) develop a gastric hypersecretion, "peptic" ulcers, and usually expire within two weeks. Shoch(2) produced ulcers in 70% of all animals when he subjected them to daily intramuscular injections of 30 mg of histamine base in beeswax and mineral oil. The average survival time was 17 days. He later produced perforated ulcers in 100% of the animals within 9 days using the same regime. Shoch pre-

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* This research was partly supported by a grant from E. I. du Pont de Nemours & Co. who also prepared the RD-11 detergent complex.

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2. Shoch, D. E., Ph.D. Dissertation Northwestern University, 1943.

vented or reduced the occurrence of these conditions in dogs by administering 0.1 g sodium alkyl sulfate hourly.

The present investigation was undertaken to produce inactivation of enzymes by detergent complexes and to study the changes in composition of gastric secretion produced by oral administration of detergent complexes. The detergent complex used as the medicant in this experiment was sodium alkyl sulfate bound to gastric mucin (Mucosulf). Dogs medicated with 900 mg of Mucosulf only twice daily showed similar changes (*i.e.* reduced incidence of ulcer, increased survival time, and altered gastric juice formation) to those produced by Shoch using nearly twice the quantity of medicant given at hour intervals.

Experimental. The investigation was begun on November 14, 1949 and terminated 216 days later. Sixteen dogs were injected with 30 mg of histamine base intramuscularly. Of the total number of dogs, 10 were injected as described for 3 days only and their gastric secretions were used as an additional control to that obtained from the other 6 dogs for the same length of time. Beginning on the fourth day 6 dogs were given orally two 900 mg doses of Mucosulf daily after each injection of histamine while the other 10 dogs were dropped from the experiment.

There was a slight transient illness in most dogs around the fourth day. Symptoms of diarrhea, melena, and anorexia lasted for about 3 days after which the dogs became apparently normal as they remained through the entire period of medication. It is possible that an ulcer may have formed but that it soon healed.

Gastric secretions were collected through an Ewald tube on the second and third day on histamine. These served as the control. After beginning medication gastric samples were taken once every 2 weeks for the duration of the experiment. On the day of collection the first gastric sample was withdrawn 3 hours after the administration of the histamine and Mucosulf. This sample was designated the residuum. The second sample was taken one half hour after the first. The stomach was emptied in each case and the volumes recorded as total volume.

Aliquots were taken from the total volume for analysis of the total available acid (in mg HCl), pepsin (in pepsin units), mucin (in mg glucose) and lysozyme. Aliquots were also taken to determine the concentration of pepsin (in units per ml of gastric juice), mucin (in mg per ml of gastric juice), lysozyme (in μg per ml gastric juice), the pH, and viscosity (relative to distilled water). The mucin was determined according to the method of Anderson, Fogelson, and Farmer (3), the lysozyme by a modified Smolelis and Hartsell(4) method, and the pepsin by the method of Anson and Mirsky(6-8).

Mucosulf medication was stopped after 165 days and within a period of one month all but 2 of the dogs expired. Autopsies showed deep erosions, hemorrhagic gastritis, and perforating ulcers indicated that death was due to peritonitis.

Discussion. Lysozyme, mucin and viscosity assays were made following Meyer's observation(5) on lysozyme and its relationship to gastroduodenal diseases. It was found that during the Mucosulf medication there was a marked reduction in the total available lysozyme in the gastric juice without any significant change in its concentration and that the concentration of mucin and the degree of viscosity increased. When medication was stopped a rapid increase in total available lysozyme occurred with no change in its concentration. Also the mucin concentration and degree of visosity were low.

In Fig. 1, 2, 4, and 5 the gastric juice components from dogs on Mucosulf therapy fall below the variation of the control therefore there is a definite decrease in the re-

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5. Prudden, J. F., Lane, N., Meyer, K., *Am. J. Med. Sc.*, 1950, v219, 291.

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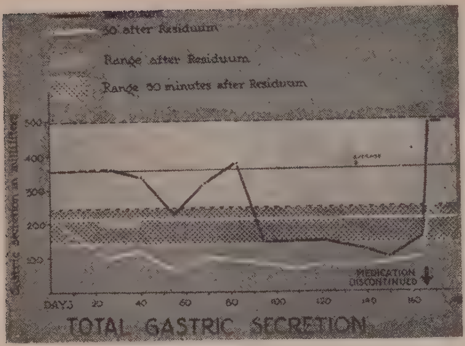

FIG. 1.

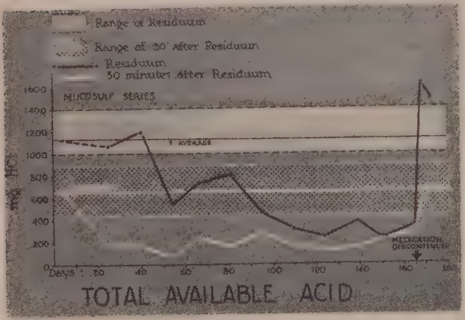

FIG. 2.

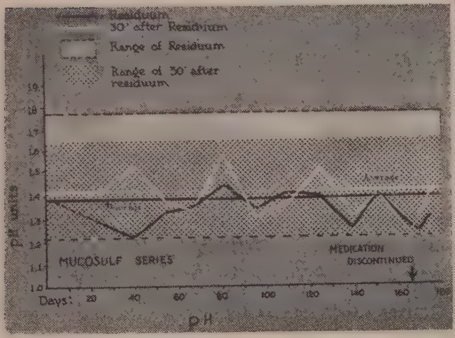

FIG. 3.

spective components of the gastric juice as a result of mucosulf therapy. However, in Fig. 3 all pH values fall within the normal variation. There was therefore, no significant change in pH as the experiment proceeded.

Each point on the figures represents the average of 6 dogs. The use of this average is considered permissible since it was shown statistically that individual variations between the dogs were not greater than the daily variations of a single dog. The solid horizontal lines on each figure represent the average of the controls while the area between the dotted lines is the range of variation.

From these data it is evident that there is a decrease in total pepsin as well as in pepsin concentration but only a decrease in total available gastric acidity not in its concentration. Therefore it appears that pepsin rather than acidity plays the primary role in experimental ulcer formation. Hence medication may well be directed toward reduction of enzymatic activity.

A second investigation is in progress using the detergent complex, sodium alkyl sulfate bound to a resin (RD-11), as the medicant.

Summary. The results obtained from mucosulf therapy of experimental ulcer for-

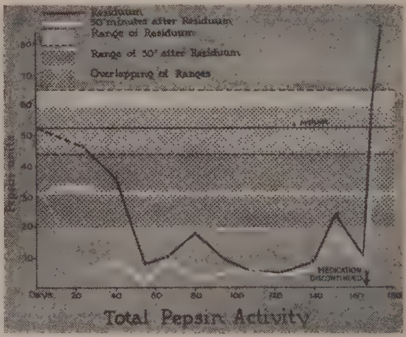

FIG. 4.

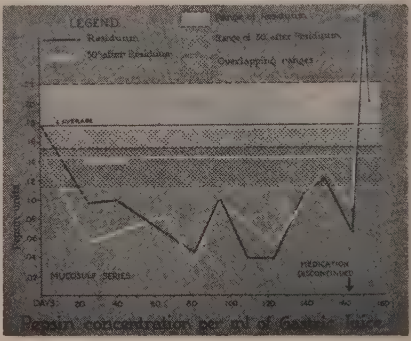

FIG. 5.

mation show that there is a decrease in total available pepsin and in pepsin concentration, but only a decrease in total available gastric acidity and not in its concentration. Total available lysozyme was decreased but not its concentration. The volume of gastric juice was decreased while its viscosity was increased. Total available mucin was increased as was its concentration.

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Effects of Cortisone, Hyaluronidase, Desoxycorticosterone, and Artisone on Experimental Serum Disease in Rabbits. (18191)

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In previous experiments it was shown that large doses of colchicine or nitrogen mustard depress or prevent the Arthus reaction in the skin and the allergic arteritis and valvulitis caused by horse serum in rabbits(1,2). As this was associated with depression of precipitin formation, and enlargement of the adrenals and shrinkage of the thymus (alarm reaction), it was postulated that the beneficial effect of the drugs in this disease was due to destruction of antibody forming cells through the mediation of the adrenal cortices(1). Recently it was demonstrated that 10 to 20 mg of adrenocorticotrophic hormone (ACTH) per day likewise depressed the Arthus reaction, allergic arteritis and endocarditis, the weight of the thymus, and precipitin formation(3). There is reason to believe that these effects are produced by the glucocorticoids, which also influence permeability of the ground substance(4), the site extensively involved in serum disease. We considered it desirable, therefore, to compare the effect in serum disease of cortisone, which causes both lymphoid involution and changes in permeability of the ground substance, with hyaluronidase, desoxycorticosterone acetate (DCA), and 21-acetoxypregnenolone (Artisone), which also alter ground substance permeability but do not affect lymphoid tissue.

Materials and methods. All experiments were performed in young male albino rabbits weighing between 1760 and 2500 g. One series of 40 rabbits was given by intravenous injection 15 ml of normal horse serum without preservative per kg of body weight. Three groups of 10 animals each received 4 mg cortisone acetate,* 2.5 mg DCA,† and 5 mg Artisone acetate‡ twice daily by intramuscular injection; the remaining 10 animals served as serum controls. Another series of 20 rabbits received 10 ml horse serum per kg. Ten of these received 6000 turbidity reducing units (T.R.U.) of hyaluronidase§ per kg of body weight (twice daily by intravenous injection); the other 10 served as serum controls. Half the rabbits of each group were killed 15 days after the first injection of serum. The other half received a second injection of serum 19 days after the first and were sacrificed 5 days later. Three additional rabbits received hyaluronidase only (6000 T.R.U. per kg twice daily by intravenous

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* Saline suspension of Cortone Acetate, Merck.

† Cortate, oil solution. We are indebted to the Schering Corp. for furnishing some of this material.

‡ Micronized aqueous suspension of 21-acetoxypregnenolone, Wyeth.

§ Hydase, Wyeth.

TABLE I. Average Size and Incidence of Arthus Reaction in Skin 24 Hr After Intradermal Inj. of 0.1 ml Horse Serum on 14th Day of Experiment.

	Positive reactions, %	Size of positive reactions, cm
Control	0	0
Serum	90	2.94 × 3.39
” + DCA	87.5	2.42 × 2.67
” + Artisone	100	2.93 × 3.43
” + cortisone	78	2.33 × 2.67
Control	0	0
Hyaluronidase	0	0
Serum	100	4.7 × 5.8
” + hyaluronidase	70	2.71 × 3.43

injection), and were killed 24 days after the initial injection. Eight rabbits served as normal controls.

Results. One each of the hyaluronidase and Artisone treated, 2 of the DCA treated, and 5 of the cortisone treated rabbits died during the course of the experiment. The Artisone and DCA treated animals died with pulmonary edema. Three of the cortisone treated rabbits showed suppurative pneumonia, pleurisy, and pericarditis; 4 revealed multiple necrosis in the liver. The Arthus reaction in the skin elicited by intradermal injection of 0.1 ml horse serum was depressed by cortisone (Table I). A similar depression occurred with DCA and hyaluronidase but not with Artisone. Proteinuria was not present in any of 22 serum or steroid treated rabbits of the first series examined on the eleventh day after the first injection of serum. A second group of 13 rabbits of this series were killed on the fifteenth day at which time 2 rabbits of 4 receiving serum only and 1 of 3 DCA treated ones had 60 to 120 mg of protein per 100 ml of urine as measured by the Exton test(5). Sixteen rabbits of the previously mentioned 22 were killed 5 days after the second injection of serum and the urine was retested. At this time only the cortisone treated animal had proteinuria (70 mg per 100 ml). The extra-capillary glomerular nephritis was mild in the cortisone treated animal and severe in the 3 other rabbits with proteinuria.

The changes in weight of the various organs following injection of 15 ml horse serum per kg of body weight are shown in Tables II and III. The tremendous increase in weight of the spleen 15 days after the first injection of serum was due largely to increased activity of the germinal centers; 5 days after the second injection proliferating plasma cells were an important factor in the enlargement. Kidneys, lungs, and heart were also markedly enlarged, apparently due to the mesenchymal changes observed in these organs.

On microscopic examination the incidence of arteritis, carditis, and glomerular nephritis was observed to be greater with 15 ml horse serum per kg than with 10 ml. It is also of interest that the albino rabbits used in this experiment had more severe serum disease than was observed in the chinchilla variety used in previous studies. Furthermore, albino rabbits receiving the 15 ml dose of horse serum showed necrotizing arteritis and extra-capillary glomerular nephritis even after the first injection (3 of 14 rabbits on the fifteenth day), whereas in 41 chinchilla rabbits such lesions were not seen after the first injection of 15 to 20 ml horse serum per kg but only after the second injection.

The arteritis was most severe in the heart, lungs, and spleen, next in the pancreas and liver, and least in the thymus and thyroid. The myocardium and heart valves were more severely involved than the endocardium and pericardium. In the lungs the small vessels were much more affected than the large ones. In the kidneys the glomeruli were especially affected. Fifteen days after the first injection the spleen was crowded with large active germinal centers, while 5 days following the second injection these were markedly depressed and abundant plasma cells were found in the spleen and liver.

The administration of hyaluronidase to serum treated rabbits caused a greater frequency and severity of arteritis and carditis, but less glomerular nephritis (Table IV). However, following the second injection of serum no significant difference was noted between the hyaluronidase treated animals and the serum controls. Precipitin ring tests were

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TABLE II. Organ Weights, and Frequency (a)* and Intensity (b) of the Various Lesions Caused by Serum.

			Heart										Lungs							Kidneys											
								Arteritis															Arteritis								
	No. of rabbits	Wt, kg	Wt, g/kg	Myocarditis a	Myocarditis b	Valvulitis a	Valvulitis b	Subacute a	Subacute b	Acute a	Acute b	Wt, g/kg	Small vessels a	Small vessels b	Large arteries a	Large arteries b	Large veins a	Large veins b	Wt, g/kg	Glomerular nephritis a	Glomerular nephritis b	Subacute a	Subacute b	Acute a	Acute b	Liver wt, g/kg	Spleen wt, g/kg	Thymus wt, g/kg	Adrenals wt, g/kg
Controls	5	2.64	2.76	60	+	0	0	0	0	0	0	4.88	60	2+	0	0	20	2+	6.74	0	0	0	0	0	0	38.6	.35	1.49	.092
15 days after first injection of serum																													
Serum	4	2.27	3.2	100	2+	50	3+	25	2+	25	5+	5.94	75	+	0	0	0	0	8.26	75	3+	50	4+	0	0	38.9	1.06	1.44	.085
Serum + D†	4	2.30	3.84	100	+	50	2+	50	2+	25	2+	5.66	100	2+	25	2+	50	+	9.66	50	3+	0	0	0	0	36.05	1.06	1.32	.082
Serum + A	4	2.13	3.47	100	2+	50	2+	50	3+	25	4+	5.30	75	3+	0	0	25	2+	9.39	75	3+	25	4+	25	4+	34.9	1.29	1.80	.079
Serum + C	8	2.08	5.64	37.5	+	0	0	0	0	0	0	6.33	33	+	0	0	0	0	11.17	87.5	2+	0	0	0	0	67.3	.44	.34	.064
5 days after second injection of serum																													
Serum	5	2.58	3.21	100	4+	100	3+	80	4+	60	3+	5.99	100	4+	100	3+	100	3+	8.68	100	4+	20	2+	0	0	39.0	1.51	1.24	.090
Serum + D	4	2.44	4.0	75	4+	75	3+	50	5+	50	4+	7.61	100	3+	50	4+	50	2+	10.63	100	3+	25	3+	0	0	38.8	1.08	.46	.105
Serum + A	5	2.58	4.29	100	4+	80	3+	80	3+	20	5+	5.06	100	3+	80	3+	80	3+	7.3	100	3+	0	0	0	0	38.4	1.05	.59	.103
Serum + C	1	2.53	4.03	0	0	0	0	0	0	0	0	5.53	100	2+	100	3+	100	2+	10.0	100	+	0	0	0	0	77.4	.40	.47	.089

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* The frequency has been expressed in per cent of animals affected, in order to facilitate comparison.

† D = DCA. A = Artisone. C = Cortisone.

TABLE III. Percentage Changes in Weight of Organs (g/kg) as Compared with Organs of Normal Rabbit.

	No. of rabbits	Heart	Lungs	Kidneys	Liver	Spleen	Thymus	Adrenals
		15 days after first inj. of serum						
Serum	4	+ 16	+22	+23	+ 1	+203	— 3	— 8
" + DCA	4	+ 39	+16	+43	— 7	+203	—11	—11
" + Artisone	4	+ 26	+ 9	+39	— 10	+268	+21	—14
" + Cortisone	8	+105	+30	+66	+ 74	+ 26	—77	—30
		5 days after second inj. of serum						
Serum	5	+ 16	+23	+29	+ 1	+332	—17	— 2
" + DCA	4	+ 45	+56	+58	+ 1	+208	—69	+14
" + Artisone	4	+ 55	+ 4	+ 8	— 1	+200	—60	+12
" + Cortisone	1	+ 46	+13	+48	+101	+ 14	—68	— 3

TABLE IV. Frequency (a)* and Intensity (b) of the Various Lesions Caused by Serum.

	No. of rabbits	Heart								Lungs				Kidneys					
						Arteritis										Arteritis			
		Myocarditis		Valvulitis		Subacute		Acute		Small vessels		Large vessels		Glomerular nephritis		Subacute		Acute	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
Controls	3	33	+	0	0	0	0	0	0	67	2+	0	0	0	0	0	0	0	0
		15 days after first injection																	
Serum	5	80	2+	20	2+	40	+	0	0	100	2+	40	2+	100	2+	0	0	0	0
Serum + hyaluronidase	4	100	3+	50	2+	25	+	0	0	100	3+	62	2+	25	2+	0	0	0	0
		5 days after second injection																	
Hyaluronidase	3	100	2+	0	0	0	0	0	0	100	4+	0	0	50	+	0	0	0	0
Serum	5	100	2+	80	4+	60	3+	20	4+	100	4+	100	4+	100	2+	20	3+	20	4+
Serum + hyaluronidase	5	100	2+	100	3+	80	2+	0	0	100	3+	80	4+	60	2+	0	0	20	4+

* See footnote, Table II.

positive with the sera of all the hyaluronidase treated rabbits (0.2 ml of a 1:3 dilution of serum overlaid by 0.2 ml of a solution containing 50 to 0.5 T.R.U. of hyaluronidase) and negative with the sera of the serum controls.

The treatment with cortisone, on the other hand, had a remarkable effect on almost all lesions (Tables II and III). It prevented all vascular changes with the exception of those in lungs and spleen in the rabbit killed 5 days following the second injection of serum. It caused a marked decrease in adrenal weight, due to atrophy of the zona fasciculata, and in the weight of thymus and spleen, due to lymphocytolysis. There was a remarkable increase in the weights of liver, kidneys, and heart, apparently due to the deposition of glycogen. The glycogen content of the liver from the animal sacrificed 5 days after the second injection of serum was more than twice that of the controls (Table V). However, glomerular nephritis was not prevented; 8 of the 9 cortisone treated rabbits had up to 4 plus nephritis, intra-capillary as well as extra-capillary.

DCA and Artisone had no remarkable effect on the histological manifestations of serum disease except that the livers and adrenals of the animals receiving DCA revealed marked extra-medullary myelopoiesis. However, following the second injection of serum, the adrenals were larger and the thymus and spleen smaller than those of the serum controls, suggesting that the drugs in the dosages used had a mild alarm reaction effect. There was also a marked drop in the glycogen content of the liver in the DCA treated rabbits (Table V). No attempt was made to determine whether the doses of DCA as large as those

TABLE V. Glycogen Content of Liver 5 Days After Second Inj. of Serum. (Anthrone method).*

	No. of rabbits	Body wt, kg	Liver wt, g	Glycogen, mg/100 mg
Control	2	—	94.3	1.94
Serum	5	2.58	100.3	1.73
,, + DCA	4	2.44	104.2	0.56
,, + Artisone	5	2.58	99.6	1.85
,, + Cortisone	1	2.53	195.7	3.75

* Seifter, S., Dayton, S., Novic, B., and Muntwyler, E., *Arch. Biochem.*, 1950, v25, 191.

used by Selye to produce adaptation disease and whether larger doses of Artisone effect the severity and incidence of serum disease.

Discussion. The depression of Arthus reaction in the skin by DCA and hyaluronidase can be accounted for by the spreading action of hyaluronidase and a similar enhancement of permeability of the ground substance by DCA(4), either of which would cause more rapid dispersion of the injected antigen. Cortisone, on the other hand, like nitrogen mustard(2) and ACTH(3), acted probably by depressing antibody formation rather than by decreasing spread through the skin, for Artisone in equi-antipermeability dose had no effect on the Arthus reaction. The absence of proteinuria on the eleventh day as compared with the positive findings during the first week of serum disease described elsewhere(6) are in agreement with our conclusion that the proteinuria during the week following the first injection of serum is due to direct damage of the glomeruli by the serum rather than to allergic glomerular nephritis. The abundance of plasma cells in the spleen and liver seen soon after the second injection of serum is consistent with the concept that plasma cells are sources of antibodies. The presence of large active germinal centers in the spleen 15 days after the first injection of serum and the marked depression of these soon after the second injection supports the observation that the activity of the germinal centers follows the formation of plasma cells and antibodies(7).

It is conceivable that the enhancement of arteritis and carditis and lessening of glomerular nephritis by hyaluronidase was due to increased permeability of the ground substance by the enzyme thereby permitting more antigen and antibody to penetrate the walls of the vessels and thus diverting some of the load from the glomeruli. The results of the precipitin ring test suggest that the failure of hyaluronidase to enhance the lesions after the second injection of serum may have been due to neutralization of this enzyme by anti-hyaluronidase. This interpretation is supported by the observation that the huge doses of hyaluronidase *per se* caused myocarditis, vascular changes in the lungs, and glomerular nephritis (Table IV).

The mechanism by which cortisone suppressed the arteritis and carditis is not completely understood. It is apparent that the demonstrated depression of antibody formation through destruction of antibody forming cells(1-3) was a major factor. Other mechanisms probably of less importance also were involved since cortisone did not prevent glomerular nephritis. This latter observation is in accord with the clinical experience that cortisone has no beneficial effect in acute glomerular nephritis in humans. It is of considerable interest that one rabbit sacrificed 15 days following the first injection of serum but not included in Tables II and III because of a broken back, showed a unilateral 6+ extracapillary glomerular nephritis, the other kidney being essentially normal. In other respects this rabbit resembled the cortisone treated ones. The small adrenals (0.141 g per kg) and thymus (0.23 g per kg) and the absence of arteritis and carditis indicate that this animal had a severe alarm reaction. The decreased arteritis and carditis after cortisone and the opposite effect after hyaluronidase suggest that generalized depression of the permeability of the ground substance also were involved in the action of cortisone. The antipermeability effect of cortisone on ground substance has been observed(4,8). The failure of Artisone to af-

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fect the serum disease lesions does not support this assumption since the doses used depress ground substance permeability as effectively as cortisone(9), but larger doses would have to be administered before the permeability factor could be evaluated. The permeability of different ground substance structures may be affected to different degrees by various steroids.

These studies seem to show that serum disease is an excellent experimental tool for evaluating the total actions of cortisone-like drugs in alleviating diseases of the ground substance. Permeability studies of the joints previously described are useful as preliminary screening, for it has been found that all effective anti-rheumatic drugs suppress permeability of the synovial membrane.

Conclusions. Cortisone in doses of 4 mg twice daily depressed the Arthus reaction in the skin and completely prevented arteritis and carditis in 8 of 9 rabbits treated with horse serum. Glomerular nephritis, on the other hand, was not prevented. The zona fasciculata of the adrenals was atrophied. The mortality rate was highest among the animals receiving cortisone. Hyaluronidase in doses of 6000 T.R.U. per kg depressed the Arthus reaction in the skin and diminished glomerular nephritis, but enhanced arteritis and carditis. These observations may be explained by the ability of hyaluronidase to weaken ground substance barriers, thereby diverting antigen and antibody from the kidneys to the general vascular bed. DCA and Artisone in the doses used had no remarkable effect on the histological manifestations of serum disease, but DCA caused a depression of the Arthus reaction in the skin possibly through its hyaluronidase-like effect upon the ground substance.

It appears that serum disease is an excellent experimental tool for comparing the total efficacy of cortisone-like drugs after preliminary screening for their anti-permeability action on ground substance.

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Histological Manifestations of Feeding Polyoxyethylene Monostearates To Weanling Hamsters.*† (18192)

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It was reported recently(1) that the ingestion of 5 or 15% of polyoxyethylene monostearate preparations (Sta-soft and Myrj) by weanling hamsters caused an increased mortality rate, a decrease in food efficiency and rate of gain as compared to similar animals fed 5 or 15% lard, or 7½% lard plus 7½% celluflour in addition to the basal ration. The experimental animals suffered a high incidence of diarrhea and other gross pathological manifestations. Similar results were obtained when other surface active agents were fed to hamsters and rats(2,3). In the present report histological changes observed in the organs of the hamsters from the investigations described previously(1) are presented.

Experimental. One animal fed either 5

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* We are indebted to Mrs. Marjorie Ismond for assistance in preparation of the slides.

† Journal Paper No. 28, American Meat Institute Foundation.

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TABLE I. Summary of Histological Changes in Hamsters Fed Sta-soft, Myrj or Lard.* (0 = no effect; 1+, 2+, 3+ = slight, moderate, and severe effect, respectively.)

	Experimental groups					Control groups†		
Duration, wk	5% Sta-soft	5% Myrj	15% Sta-soft	15% Myrj	Total	5% Lard	15% Lard	Total
				Duodenum				
2	3+	lost	0	3+		0	0	
4	0	0	2+	2+		0	1+	
6	1+	1+	2+	3+		0	1+	
8	1+	1+	1+	1+		0	1+	
10	2+	0	0	0		0	0	
Frequency	4	2	3	4	13/19	0	3	3/10
Mean intensity	1.75+	1+	1.7+	2.25+	1.8+		1+	1.0+
				Ileum				
2	2+	3+	0	1+		0	0	
4	3+	3+	3+	3+		0	1+	
6	3+	3+	2+	1+		1+	1+	
8	3+	3+	3+	0		1+	0	
10	3+	3+	3+	2+		1+	1+	
Frequency	5	5	4	4	18/20	3	3	6/10
Mean intensity	2.8+	3+	2.75+	1.75+	2.6+	1+	1+	1.0+
				Liver				
2	0	1+	2+	0		0	1+	
4	1+	1+	0	1+		0	0	
6	1+	2+	3+	2+		0	1+	
8	3+	2+	2+	3+		0	1+	
10	2+	3+	3+	3+		0	0	
Frequency	4	5	4	4	17/20	0	3	3/10
Mean intensity	1.75+	1.8+	2.5+	2.25+	2+		1	1.0+

* See text for effect on other tissues.

† The duodenum, ileum and liver from animals fed either 5 or 15% lard with restricted food intake, or 7½% lard plus 7½% celluflour, revealed damages of frequency and intensity comparable to those of animals fed 5 and 15% lard (fed *ad libitum*). Also, no injury of any kind was found in the kidneys.

or 15% lard, Myrj or Sta-soft was sacrificed at 2 weeks and additional animals were sacrificed at 4, 6, 8, and 10 weeks [details of diets, etc. see(1)]. In addition, other control animals (5 or 15% lard—food restricted, and 7½% lard + 7½% celluflour) were sacrificed at 6 and 10 weeks. The animals used for histological study were selected on the basis of sex, general performance typical of the group, initial weight, etc. They were anesthetized with ether, decapitated, bled, and opened by an abdominal incision. Standardized sections of duodenum, ileum, rectum, liver, and one kidney (left) were removed. In addition to these organs, 5 testes (greatly reduced in size) from animals fed the test products were examined. A section containing the joined heads of the femur and tibia from animals studied in the 10-week period was also obtained. Tissues were fixed immediately in Zenker-formol for 8 hours, processed by the standard celloidin method, cut at 10 μ, and stained with Hematoxylin and eosin-azure II (Maximow). The organs from each animal were imbedded in one block. In the majority of cases 2 slides, each containing 3 consecutive sections, were made for the tissues from each animal. For the testes, serial sections of the entire organ were made.

Results. The results are summarized for the duodenum, ileum, and liver in Table I in order to show both the frequency and intensity of cases which definitely deviated from the normal. *General observations*: (A) Marked histological changes in the tissues were observed when 5 or 15% Myrj or Sta-soft was fed. (B) Deleterious effect of the test products used (Sta-soft and Myrj) was similar. (C) No appreciable increase in the extent of damage was found for animals fed the diets from 2 to 10 weeks; some evidence for acclimatization occurred in certain cases. (D) Little evidence for proportionately greater damage due to ingestion of the higher level of the test compounds was apparent. (E) No difference in effect of these products due to sex of the animals was observed. (F) Significantly less damage, both in frequency and in extent, was observed for the animals fed lard.

A description of the specific injuries observed follows:

Duodenum and ileum. The histological damage in these 2 organs was of a similar nature, but varied in intensity in different cases (Table I). For both organs, the cases which showed no or slight effect were from animals fed lard, and those with moderate and severe injuries were from animals fed either Sta-soft or Myrj. The injury was principally a necrotic erosion of the mucosa, and in severe cases, half of the thickness of the entire layer of mucosa was sloughed off (Fig. 1 and 3). The erosion of the ileum usually was more intensified on one side, presumably that in contact with the intestinal contents (Fig. 2). Other injuries common to both duodenum and ileum were frequently congested villi with fully loaded capillaries and leukocytic infiltration. Such congestion might increase the amount of blood lost. Blood was detected in the intestinal contents of some of the animals fed the test products(1).

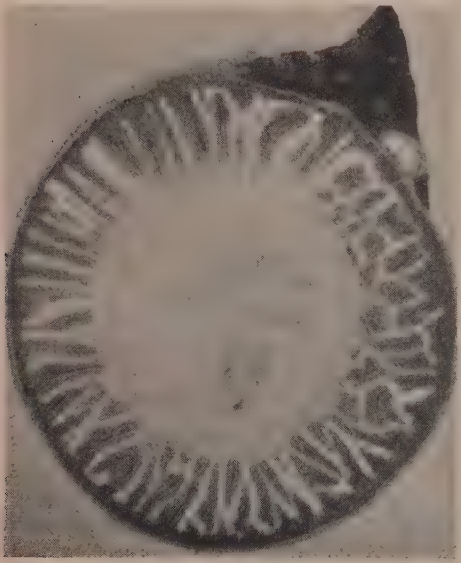

FIG. 1.
Duodenum; 5% Sta-soft, 2 wk; showing severe mucosa erosion reducing the normal thickness to half. ×24.

Liver. Injuries to the liver consisted of necrotic lesions of relatively small areas which occurred peripherally (hemorrhagic) but sometimes more deeply located. The necrotic hepatic cells were pyknotic, had a highly granular type of cytoplasm (Fig. 4) and some of the inclusions stained green with Azure II. The blood vessels and bile ducts appeared normal except for an apparent increase in blood volume, since the central veins were several times the diameter of the veins from animals fed lard. Sinusoidal congestion was observed particularly in the periphery (Fig. 5), which would likely lead to hemorrhage. As in the duodenum and ileum, leukocytic infiltration along congested sinusoids occurred frequently.

Other tissues. The rectum and myeloid tissue of bone marrow were found to be normal for all animals studied. Injury to the kidney was noted in 5 out of 20 hamsters fed either Sta-soft or Myrj. These included 2 cases evaluated as 1+, 2 cases as 2+ and 1 case as 3+. The main histological changes included deposition of an amorphous substance which stained a greenish blue inside the convoluted tubules. This substance presumably had filtered through the glomeruli of the renal capsules, but later was deposited in the uriniferous tubules (Fig. 6). Some of the renal capsules were both congested and disintegrated. This may be caused by a shrinkage of the cortex as indicated by the nodular appearance of the contour of this kidney (Fig. 6). Some of the tubules of Henle's loop were dilated and filled with a debris largely composed of the same amorphous substance and dead cells (erythrocytes and epithelial fragments). These changes are similar to those observed when large amounts of pteroylglutamic acid were injected

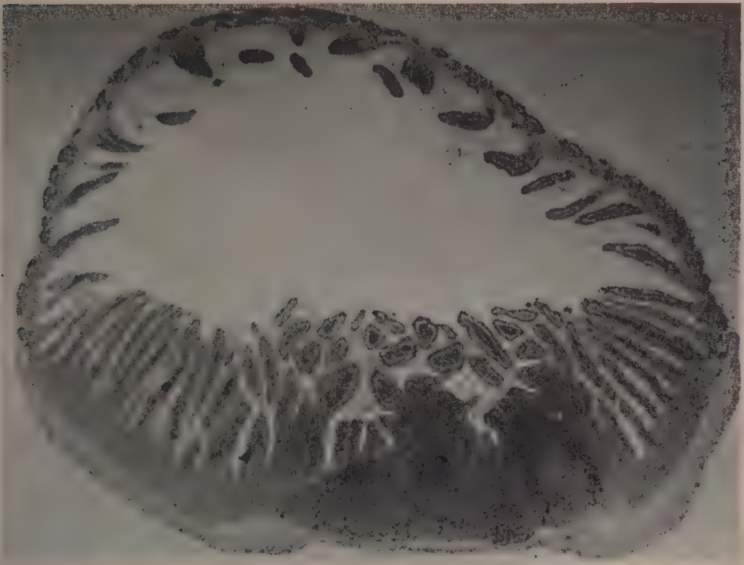

FIG. 2.
Ileum; 15% Myrj, 4 wk; showing severe injury to the mucosa of half the circumference. ×37.

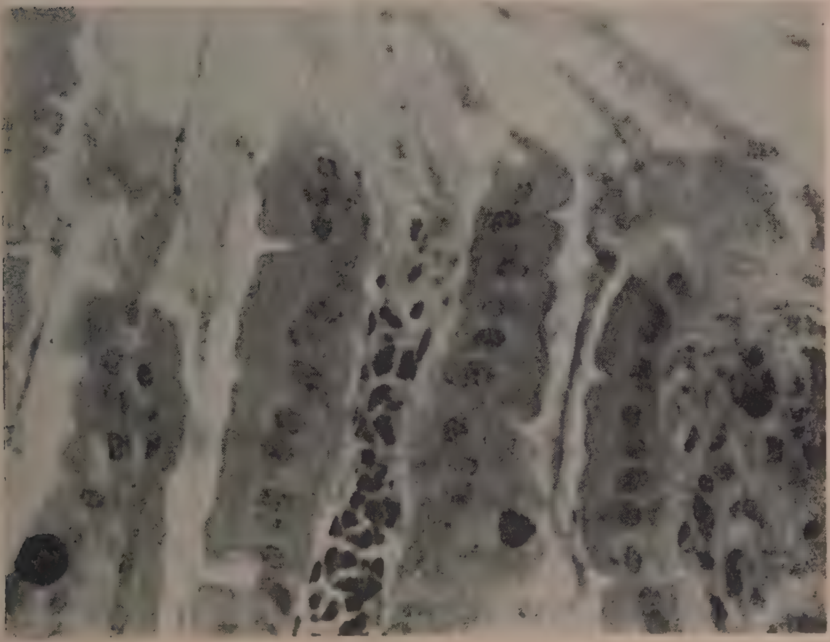

FIG. 3.
Duodenum; 5% Myrj, 8 wk; showing eroding tips of villi highly magnified (2 goblet cells as 2 dark spots). ×550.

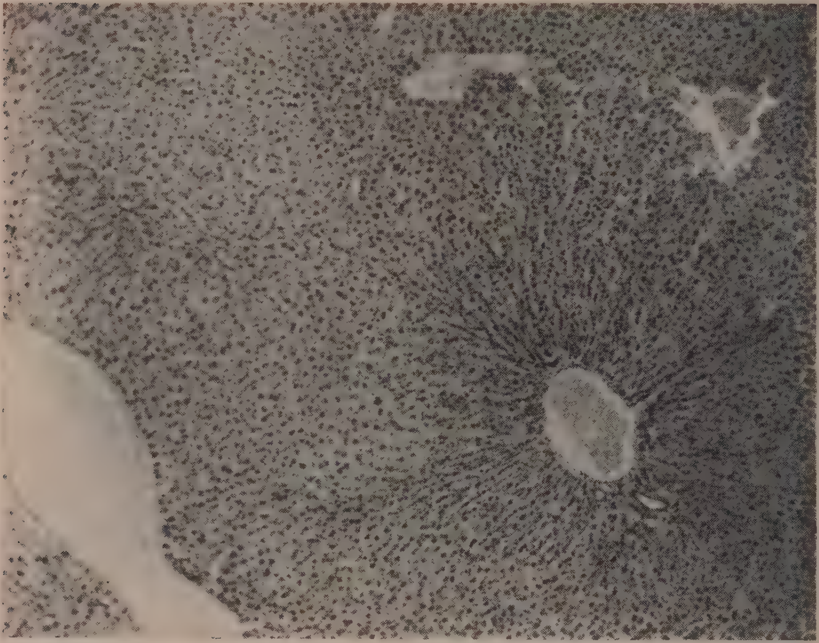

FIG. 4.
Liver; 15% Sta-soft, 10 wk; showing two intralobular necrotic lesions (upper right), one distended central vein of a lobule filled almost completely with blood cells, and one greatly distended interlobular vein (lower left). Cells of the lobule are granular and pyknotic. ×65.

into guinea pigs(4). No damage was observed in the kidneys of the animals fed lard (total of 16 animals examined).

The five testes (one each from animals fed 15% Myrj for 2, 4, and 10 weeks; 5% Myrj for 10 weeks, and 5% Sta-soft for 10 weeks) ranged in size from 1/5 to 1/3 of those from the control animals.‡ In 4 of the 5 cases, little or no spermatogenetic activity was observed in the seminiferous tubules. The lumen was either empty or contained material which resembled the remnants of spermatozoa. Normal spermatogonia and spermatocytes with occasional mitotic figures were present in these tubules. This condition suggested that spermatogenesis was abruptly halted at the level of secondary spermatocytes. Both the ductuli efferent and epididymis tubules were packed full of spermatocytes, but no spermatids or spermatozoa.

Discussion. Judging from the number of affected cases, the organs range in the order of ileum, duodenum, liver, and kidney in decreasing susceptibility to the effect of polyoxyethylene monostearates (Table I). This may mean that the greatest toxicity resulted from direct contact with the cells; and affords an explanation of why no damage was observed for the tissues of the rectum. The duodenum, which showed severe damage in animals sacrificed at 2 weeks, nevertheless, definitely showed a smaller amount of damage in the animals that were sacrificed later in the study. Both ileum and liver reached the height of damage for the animals sacrificed at 4 weeks and then remained at this level for the animals sacrificed at 6-10 weeks without noticeable acclimatization. The ef-

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4. Dawson, J. R., Jr., Woodruff, C. W., and Darby, W. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 646.

‡ Autopsy findings showed that in 3 cases the testes were approximately 1/10 the size of those from animals fed lard.

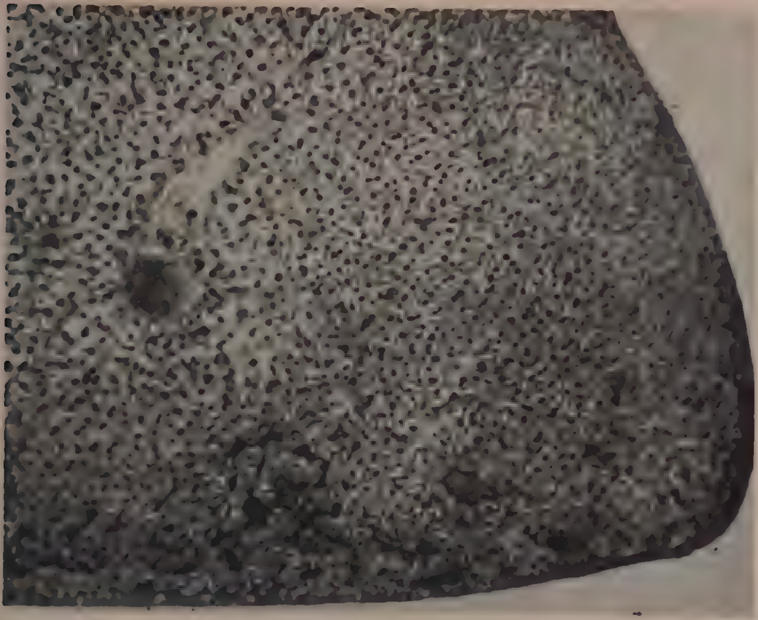

FIG. 5.
Liver; 5% Sta-soft, 10 wk; showing peripheral sinusoidal congestion. ×120.

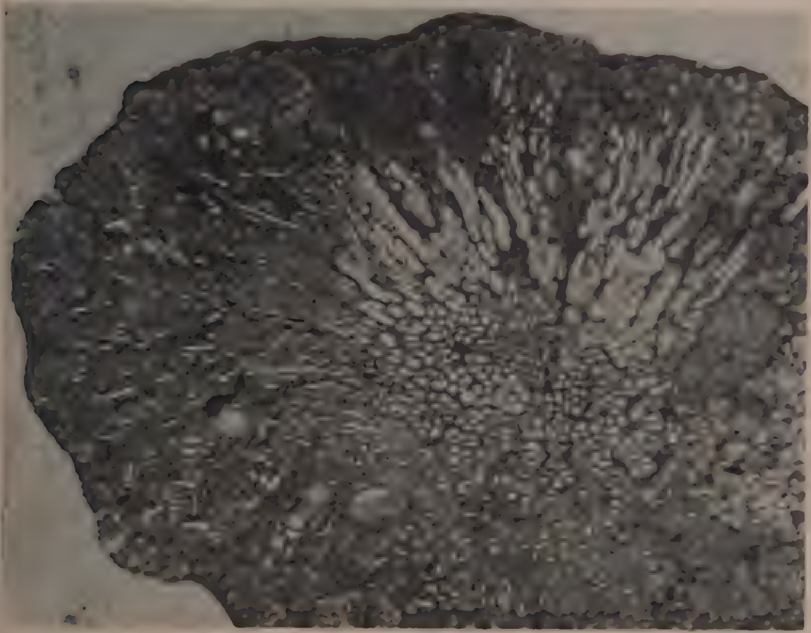

FIG. 6.
Kidney; 15% Myrj, 8 wk. Note the nodular surface and other pathological symptoms. ×20.

fects noted in the kidney were observed after the animals were on experiment for a longer time. Undoubtedly some of these trends may be attributable, in part, to the fact that the most susceptible animals died in the early phases of the study and consequently were not available for histological study.

Summary. A histological study of the duodenum, ileum, rectum, liver, kidney, testis and bone marrow of weanling hamsters fed on a basal diet containing 5 or 15% polyoxyethylene monostearates (Sta-soft or Myrj) or lard was conducted. Marked changes were observed in the duodenum, ileum, liver, kidney, and testis of hamsters fed Myrj or Sta-soft after 2-10 weeks on experiment. Histological changes observed in the small intestine, particularly the ileum, consisted of a severe erosion of the mucosa. Necrosis of the liver, decreased spermatogenetic activity of the testis, and tubular degeneration of the kidney were also observed.

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Studies on Urinary Histamine in Chronic Myelocytic Leukemia.*† (18193)

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It is now well established that there are increased amounts of histamine in the blood of patients with chronic myelocytic leukemia (1-5), and that the histamine resides mainly in the cells of the granulocytic series which are beyond the blast stage of development(5). Since blood histamine levels in chronic myelocytic leukemia as high as 25 μg/ml (250 times normal) are found, and since the turnover of white blood cells is probably rapid(6) an investigation of the fate of this potentially active material seemed of interest. It has been shown that considerable amounts of histamine are excreted in the urine(7). In man this is conjugated, probably in the form of acetyl histamine. It has also been shown that histamine base fed by mouth to dogs is excreted in the urine in the form of acetyl histamine(8). The present studies were initiated to determine whether or not the endogenous blood histamine was removed by this urinary route in patients with chronic myelocytic leukemia.

Methods. Blood histamine was estimated according to the method of Code(9) using the terminal ileum of the guinea pig as the assay object. Urine histamine was determined following the bioassay procedure of Anrep *et al.*(7) with the modifications that charcoal adsorption with subsequent elution was eliminated and the amount of alumina was increased from 2 g to 5 g for 5 ml of urine in

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† The authors wish to express their appreciation to Mr. Charles Kim for valuable technical assistance in performing the histamine determinations and to the many members of the staff of Wadsworth General Hospital, Veteran's Administration, Los Angeles, whose cooperation made it possible to obtain the specimens.

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TABLE I.

Subject	WBC, ×1000	Blood histamine, μg/ml	Fasting urine histamine, μg/hr
M. (8-19-49)	180	6.5	19.8
(4-25-50)	105	15.0	99.0
P. (3-20-50)	421	25.0	84.7
(3-27-50)	310	15.0	25.6
F.	59	8.9	28.6
T.*	35	0.31	9.1
H.	16	0.15	13.1
J.	32	0.59	7.8
T. (6-9-50)	712	8.5	26.0
(6-14-50)	695	7.2	14.0
W.	46	N.D.†	11.2
Avg		8.7	30.8

* Polycythemia vera.
† No determination.

order to adsorb all the guinea pig ileum relaxins present in urine. Because it has been shown that the absorption of food, particularly meat, elevates the urine histamine levels (7), urine collections were made between 6 and 7:30 a.m., the subjects not having eaten since the previous evening meal.

Results. The urines of 25 fasting normal adults were examined and urine histamine excretions were found to range from 5.7-68.1 μg/hour with an average of 27.0 μg/hour. Normal blood histamines in this laboratory average 0.075 μg/ml with a range of 0.030-0.11 μg/ml(5).

The data for seven patients with chronic myelocytic leukemia and one patient with polycythemia vera are shown in Table I.

It is seen that while blood values many times the upper normal limit occur in patients with chronic myelocytic leukemia the fasting urine values are quite similar to the normal average and range.

Comments. If the observations made in this laboratory indicating that the life span of the circulating granulocytes is 24 hours or somewhat less(6) are of the proper order of magnitude, then the average patient in this group (assuming 5 liters as the average blood volume) has 5000 x 8.7 μg or 43.5 mg of histamine turned over from this source in this period. This is about 100 times greater than in the normal subject. With an average fasting urine histamine excretion of 30.8 μg/hour it can be seen that only 0.73 mg of histamine would leave the body through the urine in one day. This large discrepancy suggests that the fate of the histamine found in granulocytic leukocytes in chronic myelocytic leukemia is probably not conjugation followed by urinary excretion, or alternatively that the leukocytes in leukemia have a markedly longer life span than experimental evidence in normal animals would seem to indicate. Endogenous histamine of this type may, like injected histamine(10), be in some way destroyed in the body and appear in the urine, if at all, only in small amounts.

Summary. 1. While the blood histamine is markedly elevated in chronic myelocytic leukemia, fasting urine histamine excretion is in the normal range.

2. Assuming a 24 hour life span for the white blood cell it is shown that conjugation and urinary excretion is not the mechanism of removing the large amount of blood histamine present in patients with chronic myelocytic leukemia.

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Presence of a Necrosin-like Fraction in the Extracts of Injured Tissue.* (18194)

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The pattern of injury in inflammation has been found to be referable to the euglobulin fraction as recovered particularly from acid exudates(1-3). This pattern can be modified by the chemistry of the irritating agent and by the anatomic location of the lesion. Nevertheless, the basic signs of injury seem to be due to the liberation by injured cells of this toxic euglobulin. These studies have received confirmation in the hands of Tanturi and of his collaborators(4). This material has been termed by the writer, necrosin(1,3). Recently it has been shown that a similar substance can be recovered from the injured tissues of the soft sea-shell clams, *Mya arenaria*(6). In the case of these invertebrates, the toxic material was also found in the euglobulin fraction of the crushed tissues, and similarly to the necrosin of vertebrate exudate, it was thermolabile. Thus, it would seem that necrosin is a substance released by injured cells throughout the animal kingdom. To verify this point further, various visceral organs of dogs were crushed. The extracts obtained were fractionated in an endeavor to determine whether the euglobulin fraction likewise contained a toxic substance not found either in the pseudoglobulin or albumin fractions of these extracts. Such findings would permit greater latitude of generalization on the liberation of necrosin whenever cells are injured. Smith and Smith have confirmed the presence of necrosin in canine exudates(5). In addition, they have pointed out the presence of a (closely) similar toxic material in menstrual blood. This is not wholly surprising for such material contains disintegrated cellular products from the endometrium combined with elements from the blood. In this connection the writer has expressed the point of view in an earlier communication that menstrual blood is a sort of modified physiologic exudative material(7).

Several dogs were killed and one of the kidneys, a sample of liver tissue, and of spleen were removed. The tissues were weighted. A volume of sterile saline equal to about 5 times the weight of the sample of tissue to be studied was added. The tissue was then thoroughly ground in a mortar to which sterile sand was also added. The saline extract was then centrifuged. The supernatant was treated with $(NH_4)_2SO_4$ at 1/3 saturation to obtain the euglobulin precipitate fraction. The euglobulin precipitate was separated from the pseudoglobulin-albumin fractions by centrifugation. These 2 fractions were then dialyzed in cellophane tubes against running tap water. After about 24 hours, the fractions were found free of sulfate ions. The respective fractions were then injected in amounts of 0.5 cc into the skin of normal rabbits. The final readings were made 18 to 22 hours later. The severity of the induced lesions was recorded. The data are assembled in Table I.

It is clear from this table that in each case the euglobulin fraction of kidney, liver, and spleen caused a pronounced local inflammatory reaction. The pseudoglobulin-albumin fraction, on the other hand, produced negligible effects. The specificity of the canine proteins hardly enters into the picture, for when the pseudoglobulin-albumin fraction of dog tissues were used on rabbits the reactions were minimal. Necrosin has been found to

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* Aided by a Grant from the National Advisory Cancer Council of the U. S. Public Health Service.

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TABLE I. Presence of an Injury Factor in Euglobulin Fraction of Various Organ Extracts.*

Rabbit No.	Euglobulin of kidney	Euglobulin of liver	Euglobulin of spleen	Pseudoglobulin-albumin of kidney	Pseudoglobulin-albumin of liver	Pseudoglobulin-albumin of spleen
4-83	2+	2+	2+	—	—	—
4-94	2+ to 3+	2+ to 3+	2+ to 3+	t to +	t to +	t to +
5-51	3+	4+	3+	+	t	t to +
1-51	4+	2+	4+	t	t	t
1-52	4+	4+	5+	t	t	t
1-54	3+	3+	3+	t	t	t
Effect of heating the extracts from 80°C to boiling						
R-155	t	t	t	—	—	—
R-160	t	0	+	—	—	—

* The number of plus signs indicates the severity of the induced cutaneous lesion by the extract.

t = trace.

To prevent bacterial contamination, the various chemical fractions were prepared in sterile glassware and injections were made under sterile precautions. [Furthermore, both euglobulin and pseudoglobulin-albumin fractions were prepared under precisely similar conditions and at the same time, thus rendering bacterial contamination of one fraction more than that of another somewhat unlikely.]

be thermolabile(1,6). In a similar fashion the euglobulin fraction of kidney, liver, and spleen were inactivated by heating between 80°C to almost boiling. This heating procedure caused coagulation of the proteins present in the extract, leaving a watery-like supernatant phase which in all cases was utilized for the injection purpose. The gross picture of the cutaneous lesion induced by the euglobulin fraction revealed severe central necrosis surrounded by an erythematous zone. The pseudoglobulin-albumin fractions manifested a mere papule with mild redness at the point of inoculation. The microscopic sec-

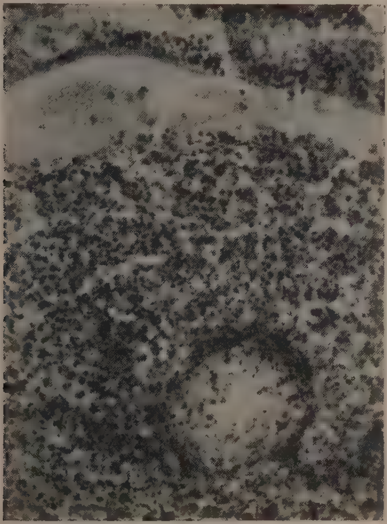

FIG. 1.

Euglobulin of kidney—22 hr and a half lesion—Abscess-like lesion with tremendous leukocytic infiltration in the skin tissue of the abdomen of a rabbit. Note the fibrinous strands in a venule and the endoarteritis of an arteriole. ×210.

FIG. 2.

Pseudoglobulin-albumin fraction of above kidney extract. 0.5 cc inj. into dermis of abdomen of a rabbit induced a relatively mild reaction with some leukocytic infiltration. Contrast this picture with the dense leukocytic infiltration caused by the euglobulin fractions (*cf.* Fig. 1). ×210.

tions substantiated the gross picture (Fig. 1). This photograph shows the dense leukocytic infiltration by the euglobulin fraction of the extracts. In some instances thrombosed lymphatics and swollen bundles of collagen were observed. In contrast, the pseudoglobulin-albumin fractions induced on microscopic study a relatively mild reaction (Fig. 2). The thermolability and the severely injurious property of the euglobulin fraction of the several organ extracts studied suggest strongly that here again we are dealing with a substance similar to the necrosin recovered from inflammatory exudate. Inflammation is a manifestation of severe cell injury. Crushing tissues mechanically performs the same operation as an irritant. This yields a similar substance from cells that have been severely injured.

Conclusions. The crushing and mashing of cells from dog kidney, liver, and spleen yields by $(NH_4)_2SO_4$ fractionation a necrotizing substance located in the euglobulin fraction of the saline extracts. The pseudoglobulin-albumin fraction of the tissue extracts do not contain this toxic substance. This substance is thermolabile. It does not seem to differ in its biological property of inducing severe cell injury from necrosin as obtained especially from acid inflammatory canine or human exudates(1,2).

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Pantothenic Acid Deficiency and Conjugation Reactions. III. Inhibitory Effect of ω-Methylpantothenic Acid.* (18195)

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The decreased acetylation of aromatic amines by the pantothenic acid deficient rat was reported(1,2) following the discovery that pantothenic acid is a constituent of coenzyme A(3). As part of our studies of factors influencing the acetylating ability of deficient rats, we report here on some of the effects of N(α,γ-dihydroxy-β,β-dimethylvaleryl)-β-alanine, termed ω-methylpantothenic acid[†] by Drell and Dunn(4). Omega methylpantothenic acid was first tested by Nease (5) and reported to show no growth-promoting activity for *Lactobacillus casei.* Drell and Dunn(4,6) noted that it inhibited the growth of a number of lactic acid bacteria. More recently, Drell and Dunn(7) have demonstrated reversible inhibition of growth with mice using this analog. Inhibition of growth of rats by the analog was prevented by simultaneous administration of pantothenic acid (8). Before the latter reports were available we decided to test this compound for its possible effects on mammals with particular reference to its influence on the acetylation of sulfanilamide by pantothenic acid deficient

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[*] Supported by a grant from the National Vitamin Foundation.

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rats before and after a given amount of calcium pantothenate. Such a procedure appears to offer a specific biochemical test for pantothenic acid analog activity and to allow results to be obtained in a short time and with much smaller amounts of the analog than would be required for growth inhibition studies.

Experimental. The acetylation test was similar to that previously reported(2). Sulfanilamide (0.8% solution) was injected intraperitoneally at 3 successive hourly intervals in amounts of 24 mg, 16 mg, and 16 mg, respectively. A 22 hour urine collection was begun immediately after the first injection. Free and total sulfanilamide was determined by the method of Bratton and Marshall(9) and the results expressed as the percentage of the total urinary sulfanilamide acetylated.

Preliminary trials with various amounts of the first sample[†] of sodium ω-methylpantothenate, given by intraperitoneal injection, indicated that the compound was capable of inhibiting the usual acetylation and growth response of pantothenic acid deficient rats[‡] given a single intraperitoneal injection of 2 mg of calcium pantothenate. With single 15, 10, 10, and 15 mg doses of the analog given on 4 successive days, 3 of 6 animals failed to respond to the calcium pantothenate given 3 hours before the start of the acetylation test. In another trial with 4 deficient rats 50 mg of the analog were given 1½ hours before injecting 2 mg of calcium pantothenate; another 50 mg of the analog were given the following day and 2½ hours later the acetylation test was started. No response to the vitamin was noted either biochemically or in growth during the following 4 days. With these preliminary data offering some basis for dosage, a more detailed experiment was performed employing the second sample[†] of sodium ω-methylpantothenate. The rats were fed diet 10-P[‡] until they were at a state of deficiency where weight gain had ceased and acetylation of sulfanilamide was reduced to 50% or less of normal values. These animals, which were approximately equal in number between the sexes, were divided into 3 groups. Group 1 received the analog for 3 days (a total of 140 mg) while group 2 received it for 6 days (a total of 230 mg); the acetylation responses with and without calcium pantothenate were noted at intervals. Group 3 served as a control and did not receive the analog. Details of treatment are given in Table I; diets which were started on a given day were continued unless otherwise noted. Results of the acetylation tests are summarized in Table II. For comparative purposes there are included the acetylation data of tests given 6 days (Day-6) and one day (Day-1) before analog administration.

It is evident that, while the analog was being administered, the usual response in acetylation following calcium pantothenate administration to deficient rats was prevented (compare Groups 1 and 3 on day 2 and Group 2 on day 6). It will be noted that there was a small increase in the per cent acetylation by Group 1 on day 2 over the acetylation in the preceding test; this did not occur with Group 2, perhaps because of the more prolonged analog administration. When acetylation was tested again (day 8 or 9) after an interval without the analog the per cent acetylation had decreased (Groups 1 and 2). Finally, the animals were tested for their ability to respond to the vitamin after a period without any analog during which calcium pantothenate was ingested and a return to control values was noted (day 15 or 20).

Discussion. Because of the failure of other pantothenic acid analogs to exhibit anti-vitamin activity in mammals, ω-methylpantothenic acid assumes special interest. Its inhibiting action on acetylation *in vivo* may be due to (a) anti-vitamin action, (b) a

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[‡] The pantothenic acid deficient diet (Diet 10-P) had the following percentage composition: casein (Labco vitamin free) 18, cerelose 66, corn oil 10, salts (USP No. 2) 4, plus vitamins added per 100 gm of basal diet: thiamine.HCl 1 mg, riboflavin 1 mg, pyridoxine.HCl 1 mg, niacin 2 mg, folic acid 0.20 mg, biotin 0.002 mg, choline 100 mg, and percomorph oil 0.12 ml. Diet 10 had the composition of diet 10-P with calcium pantothenate added at a level of 5 mg %.

TABLE I. Schedule of Treatment of 3 Groups of Rats in Testing Effect of Sodium ω-Methylpantothenate

Day	Group 1	Group 2	Group 3
0	25 mg analog*; diet 10-P + ½% analog†	As in Gr. 1	H_2O; diet 10-P†
2	9 a.m.-25 mg analog	As in Gr. 1	9 a.m.-H_2O
	11 a.m.-2 mg Ca pantothenate	11 a.m.-H_2O	As in Gr. 1
	2 p.m.-acetylation test	As in Gr. 1	As in Gr. 1
3	Diet 10-P	Diet 10-P + ½% analog*	As in Gr. 1
6	—	As in Gr. 1 on Day 2	—
8 or 9	Acetylation test	As in Gr. 1 "	As in Gr. 1
10	2 mg Ca pantothenate; diet 10	As in Gr. 1 "	As in Gr. 1
15 or 20	Acetylation test	As in Gr. 1 "	As in Gr. 1

* The 25 mg of Na ω-methylpantothenate, 2 mg of Ca pantothenate and water were inj. intraper. in volumes of 1 ml.

† Restricted to 6 g daily.

TABLE II. Influence of Sodium ω-Methylpantothenate on Acetylation Response to Calcium Pantothenate by Pantothenic Acid Deficient Rats.*

Group No.	No. of rats	Day†					
		—6	—1	2	6	8 or 9	15 or 20
1	8	12.6 ± 1.04‡	13.2 ± 1.02‡	18.0 ± 1.18§	—	12.3 ± 1.24	33.4 ± 1.00
2	6	16.5 ± 2.41‡	17,9 ± 2.72‡	15.9 ± 2.04‖	16.9 ± 1.55§	10.6 ± 1.83	34.9 ± 1.82
3	7	17.4 ± 1.40‡	17.4 ± 0.81‡	31.0 ± 1.13¶	—	29.6 ± 0.97**	35.1 ± 1.20

* Mean ± stand. dev. of the mean of the % acetylation of sulfanilamide excreted in acetylation test.

† See Table I for schedule of treatment.

‡ No treatment.

§ Analog and vitamin given.

‖ Analog given.

¶ Vitamin given.

** Only 4 of the 7 rats of group 3 were tested at this time.

generalized toxic effect, or (c) a specific toxic effect on some phase of acetylation not directly involving pantothenic acid. While possibilities (b) and (c) cannot be ruled out at this time, the analog mechanism is probably the operative one. If this is correct, then when given prior to the vitamin, ω-methylpantothenic acid is either interfering with the adequate formation of co-enzyme A or its proper association with its apoenzyme. The mechanism of action of the analog will be clarified by further study of the concentration relationships between the analog and the vitamin in affecting acetylation and growth and the concentration of coenzyme A in the tissues.

Summary. An analog of pantothenic acid, the sodium salt of N(α,γ-dihydroxy-β,β-dimethylvaleryl) β-alanine (termed ω-methylpantothenic acid) has been found to prevent pantothenic acid deficient rats from responding normally to calcium pantothenate as measured by an acetylation test using sulfanilamide. When 140 mg of the analog were administered over a period of 3 days only a small response was noted; 230 mg over a six-day period prevented any response. After the analog was withdrawn and pantothenic acid administered, the animals exhibited a marked increase in acetylation.

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Reversible Inhibition of the Beat in Heart Fragment Tissue Cultures by Desoxycorticosterone. (18196)

Ivor Cornman. (Introduced by P. K. Smith)

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In an effort to demonstrate an effect of adrenal cortical hormones on isolated living tissues, the embryonic heart was chosen because its action is readily observable and experimentally reversible. An embryonic heart can be divided into many fragments, 5 to 15 of which will pulsate and will quickly respond to changes in the culture medium. With potassium, for instance, the concentration can be gradually increased until any chosen percentage of the fragments stop beating or until a threshold is reached at which all fragments are blocked. A culture in this state of delicately suspended activity quickly responds to agents antagonistic to potassium. Thus it should provide a sensitive indicator of antagonism between desoxycorticosterone (DOC) and potassium if this antagonism operates at the cellular level. It is well known that potassium alters the activity of the heart *in situ,* whether the high potassium level results from injection(1,2) or from adrenal insufficiency(3,4). The accepted view assigns DOC effects to systemic decrease of potassium resulting from increased potassium excretion. Nevertheless, the possibility of direct effects of DOC on heart tissue and of DOC-potassium antagonism within tissues appears worth testing.

Materials and methods. Hearts of chick embryos 9-11 days old and of newborn mice or fetuses 9-14 mm in length, were cut into fragments, cemented in roller tubes with chicken plasma, and supplied with 1 ml of balanced salt solution (Gey's modification of Tyrode's). The pulsations were recorded during a 1-4 hour stabilization period before addition of isotonic KCl (1.08%) or the steroids suspended in balanced saline. Where serum was added, it was usually Difco lyophilized human placental serum. About 100 chick hearts and 150 mouse hearts were used in these experiments, providing a total of about 2500 pulsating fragments. Moreover, most fragments continued to beat for several days, so each could be tested for recovery and for uniformity of response even after long exposure. All results reported here have been obtained with both avian and mammalian heart fragments. Upjohn cortical extract, DOC (Ciba), DOC (Delta), DOCA (Delta), cortisone (Merck), cortisone acetate (Merck) and epinephrine (Parke, Davis) were tested on untreated hearts and on potassium-blocked hearts. The Ciba and Delta DOC behaved differently(5). Doses refer to the Delta product except where Ciba is specified. The steroids were dissolved in acetone and then suspended in saline. The concentrations reported here are all final levels, reached by gradual addition of the experimental agent over a period of hours. A culture was scored as blocked only when all the fragments had stopped beating, and as near-block when less than 20% continued beating. Stimulation could be detected as increase of speed and amplitude in beating fragments and as initiation of beat in fragments which had been quiet during the 1-4 hour preliminary observation.

Results. Action of cortical steroids or epinephrine on the potassium-blocked heart. Chick and newborn mouse hearts were blocked at final concentrations of 2%-7% of isotonic KCl, while fetal hearts required 7%-19%. The effect was readily reversible on return to balanced salt solution, even when the

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hearts had been immobilized for 12 hours. When hearts immobilized by KCl received a final concentration of 2%-5% cortical extract without diluting the KCl, some fragments began to pulsate within a few minutes. However, the extract is known to contain traces of epinephrine, and when ½ ppm. epinephrine was added alone, the fragments responded in a similar way. DOC at 0.005-0.067 mg/ml, its acetate at 0.015-0.077 mg/ml (in excess of saturation) or cortisone acetate at 0.025-0.166 mg/ml (in excess of saturation) administered immediately after or several hours before addition of potassium did not diminish the depression by potassium.

Suppression of heart beat by DOC. DOC by itself first stimulated and then depressed pulsation. Chick and newborn mouse hearts were blocked at 0.009-0.03 mg/ml in balanced saline, while 9-14 mm fetal mouse hearts required 0.046 mg/ml for a near block. In the absence of potassium (isotonic NaCl + $CaCl_2$) 0.005-0.01 mg/ml was sufficient to stop chick hearts, and 0.01 mg/ml blocked fetal mouse hearts. Blocked heart fragments slowly resumed pulsation when DOC was removed and replaced with balanced saline. In 7%-9% serum 0.1-0.2 mg/ml DOC was required to block hearts. Fragments which had been stopped by DOC recovered when serum was added up to 5%-10% without altering the concentration of DOC. Suppression and recovery could be repeated by adding more DOC and then more serum. This technic was employed to assay serum from different sources. There was no significant difference between lyophilized human placental serum, normal adult serum, or adult serum obtained 2 hours after adrenalin injection. Several influences could play a role in reversing the DOC effect: pH, general improvement in the tissue, adsorption or specific metabolic antagonism. Lyophilized serum shifts the cultures from the usual pH 7.4-7.6 to 8.2-8.6. However, DOC will still inhibit in cultures brought to pH 8.6 with $NaHCO_3$, and cultures in 8% serum acidified to pH 6.6 with CO_2 are resistant to DOC. Activity and survival of heart fragments is better in the presence of serum, but cultures transferred from serum to balanced saline, quickly scored and then poisoned with DOC fell well within the range of doses required to inactivate cultures maintained for 24 hours in balanced saline before dosage. To determine the site of the serum effect, cultures were dosed with enough DOC to quiet them in the presence of 8%-9% serum, but the serum was not added until most fragments had become inactive. When the serum was added, some of the fragments resumed their beating and then stopped again.

Effects of DOCA, cortisone, cortisone acetate, epinephrine or acetone. DOC acetate up to 0.038 mg/ml (persistent crystals visible within the culture tube) or cortisone alcohol or acetate up to 0.15 mg/ml were without effect on the beat. Cortisone up to 0.25 mg/ml did not decrease the inhibitory effect of DOC, and at the higher doses possibly had a slight synergistic influence. Epinephrine at 1:100,000 brought recovery lasting a few minutes to a few hours in 1 or 2 fragments per tube. Acetone at 1% did not affect the pulse. In most DOC-treated cultures the acetone carrier did not exceed 0.2%. The phenomena of blocking, recovery in serum, temporary recovery in DOC overdose, and non-antagonism between cortisone and DOC were verified with Ciba DOC. Chick and newborn mouse hearts were inactivated at 0.05 mg/ml, and recovered upon addition of 8%-9% serum, but responded poorly to 1:100,000 epinephrine.

Discussion. Resuscitation of potassium-blocked hearts by cortical extract so closely resembles the response to epinephrine at concentrations which can be present in the cortical extract, that it must for the present be regarded as an epinephrine effect(6). DOC exhibits neither a specific antagonism for potassium, nor antagonism of the sort exerted by aliphatic lipids(7). Nor is the DOC effect mediated by potassium, for DOC is more effective in the absence of potassium. Moreover, epinephrine acts upon most fragments subdued by potassium, but rarely—perhaps not significantly—on fragments in-

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activated by DOC. These effects of DOC on cultured heart fragments probably do not represent any normal function of the steroid within the body, although Cleghorn *et al.* have indicated the possibility of a specific beneficial influence of DOCA on the heart. Adequate DOCA therapy will abolish the cardiac arrhythmia of adrenal insufficiency(8) although maintenance doses sufficient to return plasma electrolyte values to normal may not improve the electrocardiogram(3). The DOC-induced inhibition of the heart fragments in culture may be related to certain systemic effects of DOCA overdosage. DOCA and other steroids act as depressants(9,10). Weakness of skeletal muscle and irregularity of heart beat induced by DOCA can be prevented by increased potassium intake(11). In cultures the heart was less inhibited by DOC in the presence of potassium. Difficulties are likewise encountered in trying to relate cortical steroid effects in the body and on cultures of kidney, where cortisone increases excretion by mesonephric tubules(12).

Serum most probably interferes with the DOC effect by adsorbing the steroid(13) although the temporary recovery of hearts in the presence of an overdose of DOC points to a possible site of counteraction within the cell. If there is an antagonist in serum, it would not appear to be cortisone inasmuch as cortisone alone will not interfere with DOC, and sera presumably high in cortisone activity (placental or post-epinephrine) proved no more antagonistic than normal adult serum. Nor could any synergist in serum be demonstrated by blocking hearts in serum and then adding cortisone. The inertness of DOC acetate may result merely from its low solubility, but could also be interpreted as evidence that only the unesterified steroid is physiologically active and that heart tissue is unable to hydrolyse it.

Summary. DOC reversibly inhibits the pulsation of mouse or chick heart fragments *in vitro*. The inhibition can be removed by serum but not by cortisone, and is more effective in the absence of potassium. DOC and cortisone have no influence upon potassium inhibition of the heart. Epinephrine will almost completely counteract potassium inhibition but revives only a few DOC-poisoned fragments.

I am indebted to Dr. Erwin P. Vollmer and Dr. Raymund L. Zwemer for their suggestions which guided these investigations. The Ciba DOC was contributed by Dr. Ernst Oppenheimer of the Ciba Pharmaceutical Products, Inc. These studies were financed by an institutional grant from the American Cancer Society, Inc., to the Helen L. and Mary E. Warwick Memorial for Cancer and Allied Diseases.

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Factors Influencing Measurement of Hyaluronidase Activity by Dermal Spread of an Indicator.* (18197)

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From the Departments of Surgery and Anatomy, University of Michigan Medical School.

The enzymatic hydrolysis of the hyaluronic acid component of connective tissue ground substance as measured by the dermal spread

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of an indicator has become a useful tool for investigation. Employing this method of study, it became apparent that there was considerable difficulty in reproducing results. Well aware that there are many biological factors incapable of definitive control, this report presents the results of a study, in which controllable physical manipulations that are operating during an investigation of the enzymatic activity were varied. These factors include: the volume injected, the enzyme content, the rate of injection and the time elapsing between injection and measurement.

Materials and methods. Twenty-three young, female Sprague-Dawley white rats were used as the test animals. The hair was clipped closely over the entire dorsum and injections were made using comparable locations on each side of the midline for control and experimental studies. Aseptic technic was maintained throughout except that there was no preliminary sterilization of the injection sites. A commercial preparation of hyaluronidase[†] was employed which assayed 500 viscosity units per cc. Because the enzyme preparation contained sufficient sodium chloride to make it isotonic when dissolved in one cubic centimeter of distilled water, 2 preparations of the indicator substance, hemoglobin, were necessary. One, to be used as the control, was made isotonic with sodium chloride; the other, for solution of the enzyme, contained no sodium chloride.

Five experimental conditions were employed (Table I). The variable in each experiment is in italic print. The injections were made at a constant rate of injection, 0.01 cc/5 sec., except where the rate of injection was the variable. The area of spread was determined by caliper measurements of the length and width of the ellipsoid. The measurements were taken as rapidly as possible through the first 15 minutes of enzyme activity. The arithmetical means of area of spread for each minute were calculated. Measurements of continued spread were made at 5 minute intervals through 30 minutes, then every 10 minutes for the first hour. After that, the interval was every half hour, until the termination of the experiment at 5 hours. A total of 1056 observations was made during the course of the entire study, averaging 8 observations for each time period in each of the individual experiments. Seventy-one per cent of all single observations fell within a range of 10 sq. mm in area from the curve representing the arithmetical means of each experiment.

TABLE I.

Experiment	Vol. inj.	Enzyme content	Time of inj.
A	0.05 cc	0	25 sec.
B	0.05 cc	*25 units*	25 sec.
C	0.05 cc	25 "	*5 sec.*
D	0.05 cc	*12.5* "	25 sec.
E	*0.1 cc*	25 "	50 sec.

Results. Fig. 1 graphically presents the results of all the experiments. The ordinate is the logarithm of time in seconds and the abscissa is area in sq. mm. There is an overall pattern of spreading characterized by two phases of activity: the first, a period of very slow spread; and the second, characterized by an accelerated rate of spread. The variations in the spreading process produced by varying enzyme content, rate of injection, and volume of injection are clearly demonstrated. Fig. 2 is a presentation of the data from all 5 experiments on a single animal. It demonstrates the uniformity of results obtained. There are quantitative differences from the means obtained in the entire study, but qualitatively the general configuration of the curves in the two graphs is identical. It is evident, therefore, that each of the experimental variables, such as enzyme content, rate of injection and volume of injected material substantially influences the area of spread at a given time after injection.

A comparison of curve C with curve B of Fig. 1 shows that with constant volume of injection and constant enzyme concentration, the more rapid the rate of injection, the larger will be the area of spreading at comparable times. The area increase is con-

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[†] Generously supplied as Alidase by Dr. Irwin C. Winter, Director of Clinical Research, G. D. Searle and Co., Chicago, Ill.

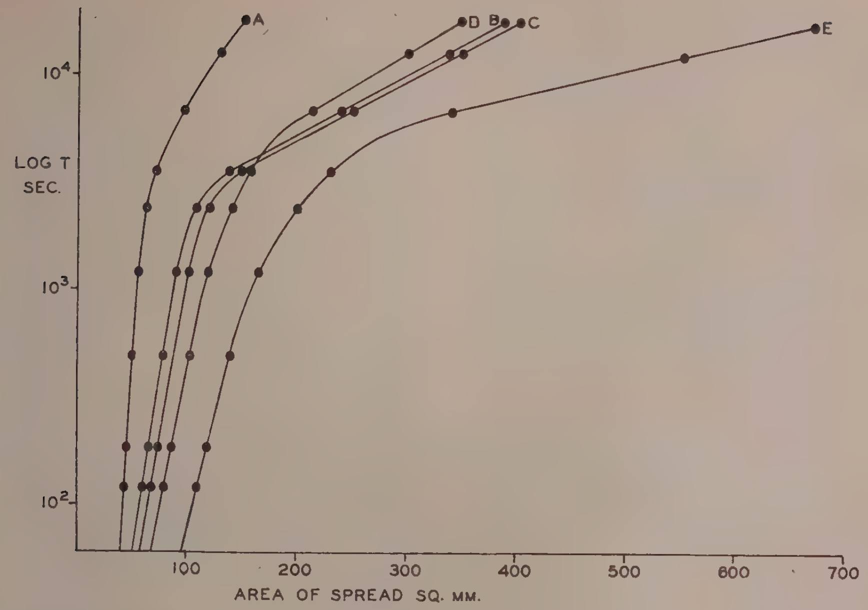

FIG. 1.

Variations in rate of spread resulting from altering certain factors: A—control; B—25 units enzyme; C—injection time 5 sec.; D—12.5 units enzyme; E—0.1 cc volume. (*cf.* Table I).

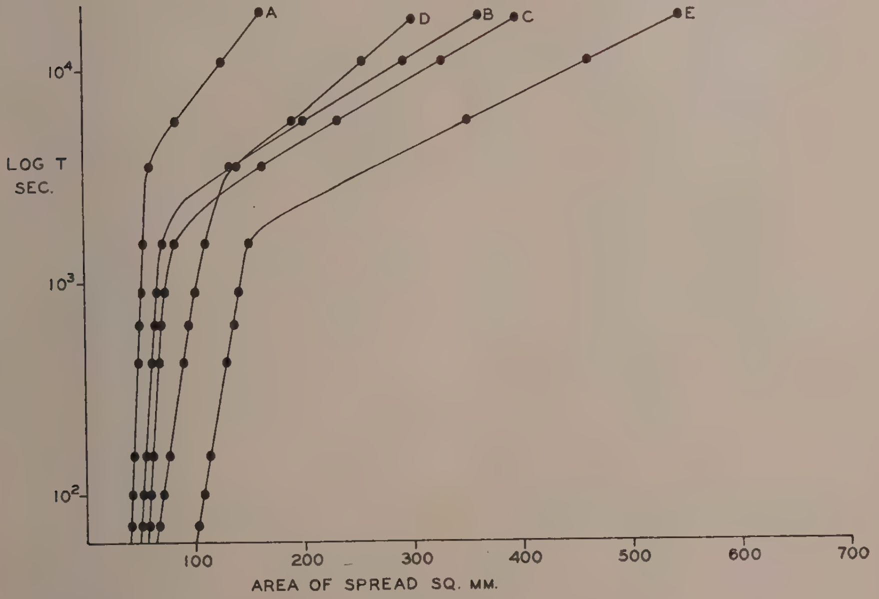

FIG. 2.

Graphic presentation of the data obtained from a single series of experiments on the same animal. (For explanation of the curves, see Table I).

sistently and uniformly greater throughout the 5 hour observation period. Curve D compared with curve B is remarkable in that with a constant rate of injection and constant volume, one-half the enzyme concentration produces a larger area of spread during the slow initial phase. The smaller area of spread in the rapid phase is the more to be expected result.

If the volume of the injected solution is doubled, but the enzyme content is constant and the rate of injection is unchanged, *i.e.*, .01 cc/5 sec. (curve E, Fig. 1), there is a much greater rate of spread compared with curve B, Fig. 1.

Discussion. In this study, no attempt was made to control age, weight, or state of the estrous cycle in the test animals. No evaluation was made of the individual animal's adrenal cortical response to the trauma of handling, operative manipulations and periods of restraint. Whether or not the regenerating hair pattern influences the dermal spread of hyaluronidase is not known and was not considered. Evans and Madinaveitea (1) have postulated on theoretical grounds that the kinetics of the spread of an indicator solution through the dermal layer might be governed by one or both of two steps. The first is a reaction between the diffusing factor molecules and the relatively nonpermeable wall of the dermal tissue. The second is the diffusion of the enzyme into the layer of solution which has been depleted of diffusing factor molecules by the reaction. The second step is supposed to be much slower than the first and is, therefore, the process which is followed experimentally. The data obtained in the present study fail to confirm such an interpretation since all the curves of activity are characterized by an initial phase of slow spread followed by a phase of more rapid spread.

The data from these experiments show that in employing the effect on the spread of an indicator by hyaluronidase as a biological measurement, certain physical factors must be carefully controlled. Failure to measure areas at uniform intervals after injection may result in considerable variation. The greatest error in reproducing comparable results arises from this single factor. For example, comparing curves A and B (Fig. 1), if A (the control) is read at 2 hours and B is read at 1½ hours, an error of 30 sq. mm (10%) obtains. Though the effect of rate of injection is consistently present, it constitutes a small error. From the shapes of curves B and C (Fig. 1), these appear to approach identical values after a period of 5 hours.

The peculiarity of a larger area of spread with a lower concentration of enzyme in the earlier phase of spreading as evidenced by curve D, Fig. 1, suggests that there may be an optimum concentration of enzyme for maximum spreading. Only when it reaches the more rapid phase does the curve conform to the usual pattern for the relationship of rate of reaction to enzyme concentration (2). Curve E, Fig. 1, illustrating the effect of increased volume of injection, shows a uniformly greater spreading effect, though the rate of injection and the enzyme content are constant.

It is suggested that the substrate concentration may be the limiting factor during the course of the slow phase of spreading. Such an interpretation would account for the experimental findings.

Summary. 1. The presence of several controllable sources of error in the study of indicator spreading by the enzyme hyaluronidase has been demonstrated. These are rate of injection, enzyme concentration, volume of the injection mass and measurement after equal intervals of enzyme activity. 2. Each factor of error singly or in any possible combination with others may materially alter the results of any investigation using this spreading as a biological measure.

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Dermal Spreading of Hyaluronidase as Influenced by Prolonged Local Treatment with Adrenal Cortical Extract.* (18198)

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The inhibition of the spreading activity of hyaluronidase by adrenal cortical extract and various adrenal cortical steroids has been reported by several investigators(1-4). Opsahl (1) and Seifter *et al.*(4) believed that adrenal hormones may inhibit hyaluronidase indirectly by increasing the resistance of the ground substance to enzymatic depolymerization. These investigators did not rule out the possibility that adrenal steroids inhibit the enzyme by direct action. Since Castor and Baker(5) have demonstrated that prolonged local treatment of the skin with adrenal cortical extract induces marked structural changes in the dermis which appear to involve the ground substance, this report is concerned with the study of the effect of pretreatment of the skin with adrenal extract on the spreading action of hyaluronidase.

Materials and methods. Eight adult male rats of the Long-Evans strain were used, 5 being treated and 3 serving as controls. For 4 months 0.1 cc of an adrenal extract (ACE) in 25% alcohol[†] was applied to the dorsal cervical region just caudal to the right ear. The controls were treated on the right side in a comparable area with 25% alcohol. In both groups, the left side received no treatment. The indicator employed was a hemoglobin solution. The final injection quantity was 0.05 cc containing 25 units of hyaluronidase[†] in an isotonic solution of the indicator. The time for the injection was 25 seconds. The animals were quieted by the subcutaneous injection of 0.2 cc of a veterinary solution of sodium pentobarbital (6.5% solution) and were anesthetized lightly with ether only for the duration of the initial injection. The hair was clipped closely from the proposed injection sites and the animals were secured loosely for constancy of position.

The manipulations of the injection procedure were controlled carefully as suggested previously(6) and the intradermal injections were made in comparable sites on each side of the animal. The measurements made were the length and width of the ellipsoidal area of spread. The first measurement was made 60 seconds after the beginning of the injection and then every minute for 15 minutes; every 5 minutes through 30 minutes; every 10 minutes through one hour and every half hour through 2 hours. A total of 736 observations was made on the 8 test animals. The increase in area for each time interval over that of the initial injection area was then calculated. The arithmetic means of all increases were determined and plotted graphically, the ordinate being the logarithm of time in seconds and the abscissa the increase in area of spread over the initial area in square millimeters. Eighty-seven per cent of all single observations fell within a range of 6 sq. mm from the plotted arithmetical means.

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[†] The authors wish to express their gratitude to Dr. Irwin C. Winter, Director of Clinical Research, G. D. Searle Co., Chicago, Ill., for the hyaluronidase (Alidase) and to Drs. M. H. Kuizenga and W. J. Haines, The Upjohn Co., Kalamazoo, Mich., for the adrenal extract. The extract was made from hog adrenal glands in 25% alcohol and 1 cc was equivalent to 0.5 mg of 17-hydroxycorticosterone as determined by the liver glycogen test.

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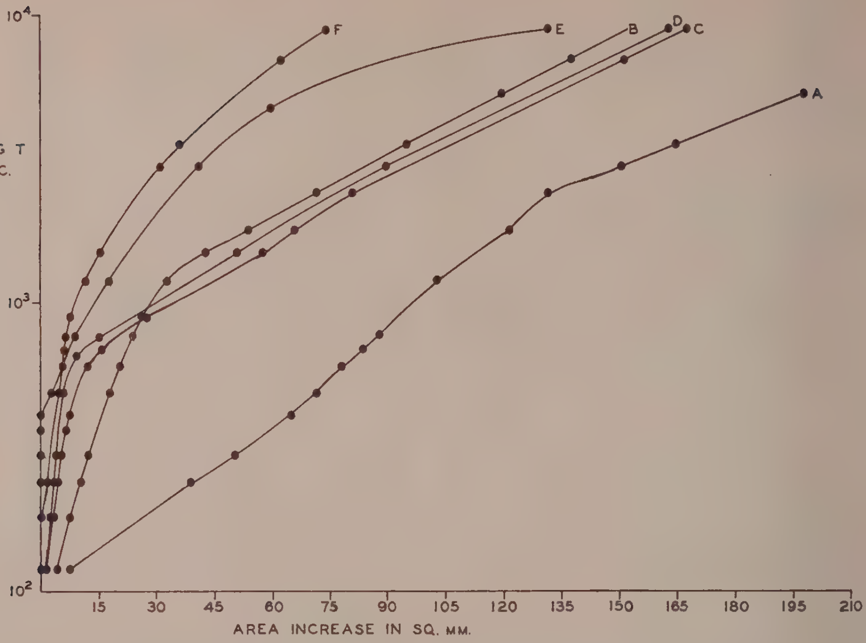

FIG. 1.

Graphic presentation of results expressed as arithmetic means: Curve A, enzyme plus indicator on treated side of ACE animals; Curve B, control side of ACE animals; Curve C, treated side of alcohol animals; Curve D, control side of alcohol animals. Curves E and F represent the observations made on a single ACE animal using indicator alone. Curve "E" is the treated side. Curve "F" is the control side.

After a period of 10 days, the experiment was repeated on a single adrenal cortical extract treated animal using indicator solution without hyaluronidase to determine the effect of local pretreatment on the spread of the indicator alone.

Results. The results are presented graphically in Fig. 1. Curve A represents the spread of hemoglobin indicator plus enzyme as influenced by the previous application of adrenal cortical extract. The initial slow phase of spreading which has been described(6) is much less distinct, resembling more a straight line function.

Curve B presents the results from the untreated side of the group of animals receiving ACE locally on the opposite side. After an initial slow phase which is intermediate between the determinations from both sides of the alcohol control animals and the right side of the ACE animals, the rapid phase of spread conforms closely to the spread of the alcohol-treated group. Curves C and D, respectively, represent the right (alcohol-treated) and left (no treatment) sides of the alcohol control animals. No significant difference is seen between the two.

Curve E is a single experiment employing an isotonic saline preparation of the indicator without enzyme on the ACE side of one animal; Curve F is the control (no treatment) side of the same animal. While no quantitative conclusions can be made from this single examination, it is readily apparent that the pattern of spreading action is distinctly different from the experiment in which the enzyme was injected (Curve A).

Discussion. The observation that pretreatment of skin with adrenal extract increases the rate and extent of spread by hyaluronidase is contrary to the reports of others(1-4) who demonstrated an opposite effect when adrenal hormones and hyaluronidase were administered concurrently. Our previous work(6) suggested that the substrate concentration serves as a limiting factor in the slow phase of spread. However, it appears that adrenal extract so modifies the connective tissue ground substance as to accentuate the spreading. From the shape of Curve A, which demonstrates a definite alteration in the initial slow phase of diffusion, one may infer that the substrate (hyaluronic acid) has been so reduced in amount and/or modified in character that the substrate is no longer as sharply a limiting factor in the enzymatic activity as on the untreated side.

It has been shown previously(6) that irrespective of the enzyme concentration and/or the volume injected, all "normal" spreading activity is characterized by an initial slow phase followed by a more rapid phase of spreading. The observation in this study that indicator alone (Curve E) does not spread as rapidly in the ACE treated area as does the enzyme plus indicator emphasizes the fact that the result is not due entirely to the extreme thinning of the dermis(5), but that there has been some structural alteration in the ground substance which renders it more susceptible to hydrolysis by the enzyme.

The significant alteration in the slow phase of spreading in Curve B suggests that there is a concurrent systemic effect on the ground substance even though the treatment always had been localized. This alteration lends further support to the contention that the action of ACE is primarily on the slow phase since no significant difference from the alcohol controls (Curves D and C) is apparent in the rapid phase.

From the foregoing conclusions, one may assume that the difference in the slow phase of spreading in the ACE-treated skin may be characteristic of the alteration in the ground substance; the exact nature of this change is as yet unknown. These observations raise the possibility that prolonged action of adrenal cortical compounds on connective tissue ground substance might be a factor in the etiology of the collagen diseases.

Summary. An accentuation of hyaluronidase activity was observed in skin which had been treated locally wtih adrenal cortical extract for 4 months. This effect was the result of a probable alteration in the connective tissue ground substance.

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Effect of Antibiotics on Mortality from Internal Radiation.* (18199)

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Rats dead of P^{32} poisoning commonly show bacterial lesions at autopsy. Because of the possibility that bacterial toxemia might play a rôle in radiation death, an investigation was made of the protective effect of antibiotics.

Methods. A total of 248 white male rats of the C. F. Wistar strain weighing about 175 g each were evenly divided into 124 treated rats and 124 untreated controls. The animals were kept in individual cages in an air-conditioned unit permitting constant temperature and humidity. Radioactive phosphorus was injected intraperitoneally in amounts ranging from 3 to 6 μc/g body weight. Streptomycin and penicillin were employed in combination, since the tissue lesions showed both gram negative bacilli and gram positive cocci. The doses were 12 mg of streptomycin twice daily and 20,000 units of penicillin every other day and these were given intramuscularly in the hind extremities. Of the 124 rats which received antibiotics, 49 were pretreated for periods ranging from 3 to 10 days, while in 75 treatment was begun immediately after injection of the P^{32}. In all instances the antibiotics were discontinued

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TABLE I. Mortality in Treated and Control Rats.

					% mortality			
Exp.	No. of rats	Dose of P32 (μc/g)	LD	Type	10 days	15 days	21 days	30 days
1	30	3.0	27	Treated	0	0	0	0
				Control	0	20	27	27
2	30	3.8	47	Treated	0	0	7	13
				Control	7	20	33	47
3	29	3.5	67	Pretreated 5 days	0	0	14	14
				Control	0	36	43	67
4	30	5.5	67	Treated	0	27	47	47
				Control	33	53	67	67
5	30	5.0	73	Treated	0	13	27	33
				Control	0	40	47	73
6	16	4.5	75	Pretreated 2 wk	0	0	50	50
				Control	0	38	38	75
7	30	6.0	80	Treated	0	53	53	60
				Control	7	53	80	80
8	30	4.5	100	Pretreated 5 days	20	73	73	100
				Control	66	100		
9	23	4.5	100	Pretreated 3 days	0	14	14	33
				Control	7	60	100	

3 weeks after the P^{32} was given, since by this time the hemopoietic tissues are in the process of recovery. Varying amounts of the P^{32} were used in order to compare the effects of antibiotics over a fairly wide range of the mortality curve. However, in keeping with past experience, a perfect correlation between mortality and dosage was not obtained. On two occasions for example, injection of 4.5 μc of P^{32} per gram body weight gave an LD_{100}, while in other experiments with 5.0, 5.5 and 6.0 μc per gram, the respective LD figures were 73, 67, and 80.

Results. 1. *Mortality.* Table I gives the dose of P^{32} and the per cent mortality at 10, 15, 21 and 30 days. In all but one experiment the mortality at 30 days was lower for the treated animals than for the controls. The difference was marked in 4 experimental groups, *i.e.,* 14 as against 67%, 33 and 73, 13 and 47, and 33 as against 100. In 4 additional groups a protective effect was evident but less marked, the per cent mortality for treated and untreated rats being respectively 47 and 67, 50 and 75, 60 and 80, and 0 and 27. In one experiment the mortality for both treated and control animals was 100%. The overall figures were 83 dead out of 124 untreated rats (67%) as compared to 45 dead out of 124 treated rats (36%). Fig. 1 shows the mortality for one experimental group and Fig. 2 gives the total mortality at intervals up to 30 days.

There was no significant difference in per cent mortality for animals pretreated with antibiotics and those treated immediately after injection of the radioactive material. The data indicate that prolonged use of the antibiotics would not have given a significantly better survival rate. All animals which recovered were observed for at least 2 months and usually for a period of several months. Eight rats, 4 treated and 4 controls, died after 30 days, *i.e.,* between 5 and 7 weeks.

2. *Survival.* The control rats began to die earlier than those treated with antibiotics. This is shown in Fig. 2 which indicates a lag in mortality for treated rats at periods of from 10 to 30 days. However, the difference in average survival time (30 days) for all untreated and treated animals dead is not impressive, *i.e.,* 15 as compared to 19 days. This is probably due in part to compression of the survival curves in the higher radiation dose groups.

3. *Morbidity.* In general loss of weight was less marked in treated than in control rats. The treated animals consumed more food and water, showed greater activity, and had distinctly less diarrhea and bleeding than the control rats.

Discussion. The mechanism of radiation death has not been established. In instances where fatal outcome occurs within a few weeks rather than days, bacterial toxemia may well be a significant factor. Chrom(2) and Lawrence and Tennant(3) emphasized the frequency in experimental radiation death of infection by organisms of the colon-aerogenes group. Bacteremia was practically constant. Miller *et al.*(4) found a high incidence of bacteremia among mice receiving total

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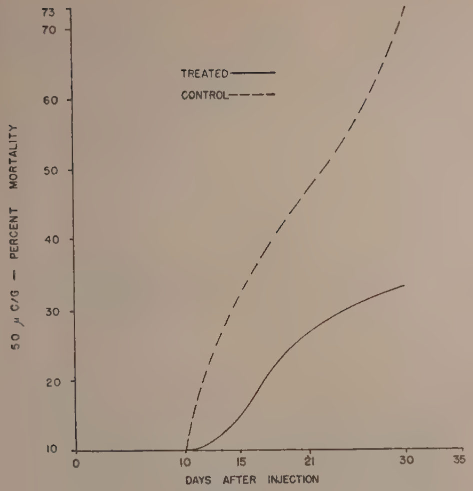

Fig. 1.

Mortality in rats receiving 5.0 μc P^{32}/g body wt.

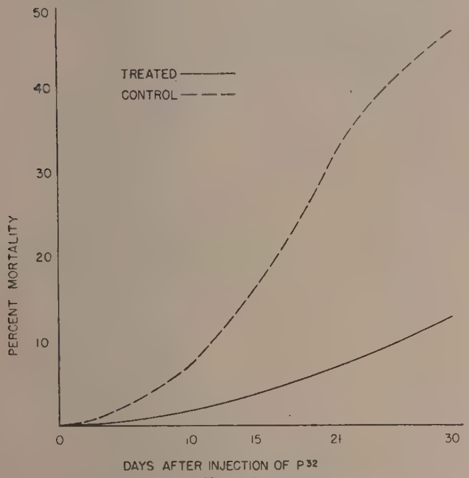

Fig. 2.

Total mortality in rats receiving 3 to 6 μc P^{32}/g body wt.

body x-irradiation of 600 or 450 r. The frequency was greatest during the period of highest mortality.

Some experimental data are available on the therapeutic effect of antibiotics in radiation injury. Penicillin was used in goats exposed to radiation during the air atomic bomb explosion at Bikini. Since no controls were available, definite conclusions could not be drawn but the data suggested that penicillin might be of value(5). Cronkite(6) treated 16 dogs with aureomycin 250 mg qid and procaine penicillin 300,000 units intramuscularly daily, beginning immediately after irradiation with 600 r. The survival time was somewhat longer but the mortality was 100% in the control and treated groups. Howland and associates(7) found that aureomycin caused almost complete alleviation of the diarrheal state in rats and dogs given lethal doses (LD_{90} to LD_{100}) of x-irradiation. Survival of life was prolonged an additional 5 to 7 days. Furth and Coulter(8) gave aureomycin to 5 dogs which received an LD_{90} (450 r) of whole body radiation. The mortality 4 months postradiation was 40% as compared to 80% for 5 control animals. Miller *et al.* (9) reported that treatment with streptomycin alone or combined with penicillin caused a significant reduction in the mortality (30 days) of mice following total body x-irradiation with 450 r.

Our data indicate that antibiotics are beneficial in the treatment of rats injured by internal radiation. The results even suggest that such therapy may be of value with radiation doses in the high lethal range (LD_{70}-LD_{100}). Further experimental investigation appears warranted. Several items need to be clarified, *i.e.*, the relative merits of various preparations, especially in combination, the most advantageous portal of entry, the effect of altering the schedule of administration, the emergence of antibiotic resistant organisms.

It should be noted that antibiotics do not alter the course of radiation injury. Damage to sensitive tissues and organs and subsequent repair are similar in treated and untreated

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rats. Moreover, treated animals which recover are equally prone to develop long term radiation effects such as the development of osteogenic sarcoma(10).

Summary. Streptomycin and penicillin in combination proved effective in reducing the mortality of rats given lethal doses of radioactive phosphorus. Morbidity was also reduced and survival time prolonged.

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Treatment of Pernicious Anemia with Crystalline Vitamin B_{12}.* (18200)

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Since the initial report by West(1) it has been suggested that one microgram of vit. B_{12} is approximately the equivalent of one U.S.P. unit of liver extract when administered parenterally to patients with pernicious anemia in relapse(2,3). Jones, Darby and Totter found that the daily intramuscular administration of 1 μg of vit. B_{12} for 22 days was followed by an excellent reticulocyte response and a satisfactory rise in hemoglobin and erythrocytes during the period of treatment (4). More recently, treatment of 30 patients with pernicious anemia in remission with vit. B_{12} concentrate in doses of one microgram daily by intramuscular injection every 3 to 4 weeks, failed to maintain optimal hematologic levels in 20 of the cases(5).

In the present study 8 persons with typical Addisonian pernicious anemia were divided into 2 groups. Four patients were treated with 1 μg of crystalline vit. B_{12} intramuscularly, daily, for 10 days, and thereafter with 14 μg every 14 days. The second group received 2 μg of vit. B_{12} intramuscularly, daily, for 10 days followed by 14 μg every week. All patients showed hyperchromic macrocytic anemia, histamine fast achlorhydria and megaloblastic bone marrow. X-ray examinations of the gastrointestinal tract, urinalyses, and blood chemistry determinations were all normal.

Results. A summary of the data is presented in Table I. The patients in the group receiving 1 μg of crystalline vit. B_{12} intramuscularly, daily, were observed for periods ranging from 53 to 108 days. The reticulocyte response in all instances was suboptimal. In only one case did the hemoglobin and erythrocytes reach normal levels, and this occurred after 108 days. The remaining 3 persons maintained stationary submaximal levels for 23 to 59 days. Patients G. and P. reached normal levels when treated with doses of liver extract which were greater than the equivalent of one U.S.P. unit a day (30 units a week for 2 months). The fourth patient, V. H., failed to report for liver extract therapy. All of the patients showed satisfactory clinical improvement as evidenced by increase in appetite, sense of well-being and gain in weight. Only one patient, V. H., showed signs of neurologic disease at the onset of treatment, and no change was observed after 93 days. The 4 patients receiving 2 μg of crystalline vit. B_{12}, intramuscularly, daily, were observed for periods varying from 48 to 250 days. The reticulo-

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* Expenses for these studies were defrayed by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

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TABLE I. Hematologic Data on Patients with Pernicious Anemia in Relapse Treated with 1 and 2 μg Vitamin B_{12} Daily.

B_{12} dose, μg/day	Patient	Initial Hb, g	Final Hb, g	Initial R.B.C. $\times 10^6$	Final R.B.C. $\times 10^6$	Retic. % peak/day	Days of observation	Remarks	Days
1	G	8	14	1.89	4.37	8.1/7	53	Blood count stationary	25
	P	5	10	1.46	3.48	10.4/10	63	” ” ”	23
	M	6	15	1.40	4.67	21.0/8	108		
	V.H.	7.5	12	2.43	3.66	No*	93	” ” ”	59
2	S	8	15	2.30	5.10	16.4/6	132	Normal blood count in	48
	Mc.	6	14	1.85	4.75	20.6/7	83	” ” ” ”	69
	Mi.	5.5	13.8	1.25	4.85	40.9/8	250	” ” ” ”	47
	F	5.5	12	1.50	4.60	33.0/9	48	” ” ” ”	48

* No retic. response.

cyte response was optimal in 3 instances and submaximal in the fourth case. Normal hematologic levels were attained in 47 to 69 days. The clinical response as regards appetite, gain in weight, strength and improvement in well being, were the same as those observed with adequate doses of liver extract or folic acid. All four cases showed moderate to advanced neurologic changes prior to treatment. No improvement, other than disappearance of numbness and tingling in one patient was observed.

The authors have under treatment 2 groups of pernicious anemia patients in remission who are receiving 14 μg of crystalline vit. B_{12} intramuscularly every 7 or 14 days. It is too early to compare these data with those presented by Meacham *et al.*(5).

Summary. (1) Four patients with pernicious anemia in relapse were treated with 1 μg of crystalline vit. B_{12}, intramuscularly, daily. Three cases showed submaximal reticulocyte responses and failed to reach normal blood levels in 53 to 93 days. Clinical improvement was observed in all instances. (2) Four other patients with pernicious anemia in relapse, treated with 2 μg of crystalline vit. B_{12}, intramuscularly, daily, showed satisfactory clinical improvement, optimal reticulocyte responses, and normal blood levels in 48 to 69 days. Symptoms of neurologic disease disappeared in one person but persistent signs were observed in all 4 cases.

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Inhibition of Phagocytosis by Aureomycin. (18201)

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Aureomycin is a widely used antibiotic in the therapy and control of many bacterial, viral and rickettsial diseases(1). It is apparently tolerated in large doses in human patients as well as in experimental animals, and aside from minor gastro-intestinal disturbances and rare allergic reactions, few untoward signs of toxic manifestations have been observed(2,3). The doses administered clinically range from 50 mg/kg/day to 500 mg/kg/day orally(4,5), resulting in blood levels of .15 μg to 20 μg per ml. Lepine and co-workers(6) reported that aureomycin as

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2. Rosanova, A. R., and Warsjewski, E., *Med. Times N. Y.*, 1949, v77, 524.

3. Harvey, J. C., Mirick, G. S., and Schaub, I. G., *J. Clin. Invest.*, 1949, v28, 987.

4. Brainerd, H., Lennette, E. H., Meiklejohn, G., Bruyn, H. B., Jr., and Clark, W. H., *J. Clin. Invest.*, 1949, v28, 992.

well as chloramphenicol (Chloromycetin) was toxic to developing cells as shown by their complete inhibition of the growth of cells in tissue culture by these antibiotics. Because of our interest in the role of the phagocytic system in the recovery from infections treated with antibiotics, an attempt to investigate the effect of aureomycin on the *in vitro* phagocytosis of bacterial cells by the polymorphonuclear leucocytes of normal human blood was initiated.

Materials and methods. The crystalline aureomycin used in this work was purchased from Lederle Laboratories as the parenteral hydrochloride salt (lot No. 7-9837). The solutions of the drug were made in M/15 phosphate buffer at pH 7.2 and used within one hour after they were made. The blood utilized was drawn from the median cephalic vein at the antecubital fossa of a normal subject. It was immediately placed in a flask containing enough saline solution of heparin (20 mg/ml) to give a final concentration of 2 mg/ml blood. The heparinized blood was used within 15 minutes after its collection. A culture of *Micrococcus pyogenes var. albus* previously used in similar studies was selected (7) because it is readily phagocytized by normal human leucocytes, and forms a smooth suspension in saline which can easily be standardized. The organisms were grown on plain nutrient agar slants for 12 hours at 37°C, washed off the slants with saline and a suspension corresponding to nephelometer standard No. 1 was made (approximately 300 million organisms per ml).

The phagocytic tests were set up as follows: 0.3 ml of M/15 phosphate buffer was added to each tube (9 mm x 75 mm), except to the control tubes containing no aureomycin which received 1.3 ml of the same buffer. One ml of aureomycin solution in the proper concentrations was added to all tubes except the controls, and following this 0.5 ml of the freshly drawn heparinized blood was pipetted into each tube. Finally, 0.2 ml of the standardized suspension of organisms was added to all the tubes. The whole procedure never took more than half an hour to be completed, and the addition of the blood and suspension of organisms was done within 5 minutes. The final volume in each tube was 2 ml. The tubes were then stoppered with corks which had been kept in distilled water for a few days to extract any substances which might have interfered with phagocytosis. The tubes were then placed in a rotating disk (15 rpm) completely submerged in a water bath at 37°C as previously described by Hanson(8). They were rotated for 15 minutes and as soon as possible after this rotation period elapsed 4 smears were made on clean slides from each tube, starting with the tube containing the highest concentration of aureomycin. The time elapsing between the end of the rotation period and the completion of making the smears was never longer than 10 minutes. To be sure that this time did not affect the number of cells showing phagocytosis, another smear was made of the first tube after it had stood for half an hour at room temperature without rotation. No further change in phagocytosis was ever noted in this tube. pH readings before and after the experimental procedure remained at 7.2. The smears were stained with the methylene blue method of Welch and Hunter(9). Four hundred polymorphonuclear leucocytes were counted under the oil-immersion lens of the microscope (100 from each of 4 slides), and the ones showing one or more ingested micrococci were recorded. In some series Wright's stain was used, but this was found to be less satisfactory than the methylene blue stain of Welch and Hunter. These experiments were always run in duplicate and were repeated several times with similar results.

Results. The results of these tests are tabulated in Table I, where the statistical

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7. Hirsch, M. M., and Novak, Milan V., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 376.

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TABLE I. Effect of Aureomycin on Phagocytosis.

Tube No.	Conc. of aureomycin μg/ml	% phagocytosis in 4 separate slides				Avg % of phagocytosis	Total No. of cells phagocytizing of 400 cells counted	Statistical analysis		
		1	2	3	4			X^2*	P*	
1	1000	10	6	8	8	8	32	190.62	$<.01$	
2	100	22	23	23	24	23	92	85.84	$<.01$	
3	10	30	33	31	35	32.2	129	35.18	$<.01$	
4	5	30	30	36	34	32.5	130	34.31	$<.01$	
5	1	35	35	41	38	37.2	149	20.02	$<.01$	
6	0.1	45	43	45	43	44	176	6.48	$<.02$	$>.01$
7	0.01	49	43	47	44	45.7	183	4.24	$<.05$	$>.02$
8	0.001	45	46	52	50	48.2	193	1.80	$<.20$	$>.10$
9	0.0001	46	53	48	50	49.2	197	1.05	$<.50$	$>.30$
10	0.00001	48	47	52	52	49.7	199	0.84	$<.50$	$>.30$
11	0.0	55	54	52	52	53.2	213			

* The chi square (X^2) values were calculated by the 4-fold table method, as described in "Statistical Methods" by G. W. Snedecor. The Iowa State College Press, Ames, Iowa (4th edition), page 199. The probability number (P) was calculated from a table given in the same book on page 190. Only values of P less than 0.05 are here considered to indicate a statistically significant difference.

analysis of the difference between the number of leucocytes showing phagocytosis in the control tubes and the number in each of the tubes containing aureomycin is also given. It can be readily seen from this table that aureomycin had a pronounced inhibitory effect on phagocytosis. The percentage of phagocytosis in the tubes containing 1000 μg of aureomycin per ml was 8, while the control tubes containing no antibiotic gave an average of 53.2%. This inhibition was less pronounced, but still statistically significant, as the aureomycin concentration was decreased to 0.01 μg/ml ($X^2 = 4.24$, and $P < .05 > .02$). This inhibitory effect of aureomycin was gradually decreased as the concentration of the drug was reduced, but even in the lowest concentration used (.00001 μg/ml) the percent of phagocytosis was less than in the control. These differences in the higher dilutions, however, are not statistically significant (Table I). The decrease in phagocytosis was marked in concentrations of aureomycin that are commonly found as therapeutic blood levels in human patients (1 to 10 μg/ml), and even below.

Discussion. The results of this work indicate that aureomycin in concentrations of 1000 μg/ml inhibited phagocytosis almost completely. This inhibition was also marked at concentrations below this level and at concentrations which are generally found as therapeutic blood levels in human patients (4,10-12). Phagocytosis was still inhibited to a significant degree when aureomycin was diluted to 0.01 μg/ml, which is far below the therapeutic blood levels which are generally recommended. Apparently the lowest concentration reported clinically is 0.15 μg/ml (4), and 20 μg/ml serum or higher are often obtained (4,11). Since there are many pathogenic organisms which are resistant to this antibiotic (13), the results reported here suggest the possibility that in those cases where the infections are due to aureomycin-resistant organisms the use of this drug may actually be harmful since aureomycin appears to interfere with phagocytosis.

Summary. Phagocytosis of *Micrococcus pyogenes var. albus* cells by polymorphonuclear leucocytes was markedly inhibited by aureomycin in a concentration of 1000 μg/ml *in vitro*. This inhibition, although less pro-

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nounced, was still significant in concentrations of the antibiotic which were considerably lower than the therapeutic blood levels ordinarily employed in clinical practice.

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Cytopathogenic Effect of Poliomyelitis Viruses *In vitro* on Human Embryonic Tissues.* (18202)

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We have previously described the cultivation of the Lansing and Brunhilde strains of poliomyelitis virus in suspended cell cultures of human non-nervous and nervous tissue (1,2). It was also noted that degenerative changes occurred in cells from cultures inoculated with the Lansing virus(1) that were more extensive than in tissues from uninoculated cultures. This phenomenon has been more extensively investigated. The most significant observations will be here summarized; a more detailed account will be published later.

Materials and methods. Suspended cell cultures. The procedures have been previously described(1,3). As routine 50 units of penicillin and 50 μg of streptomycin per cc have been included in the medium. *Plasma hanging drop cultures.* Fragments of tissue from suspended cell cultures were explanted in plasma hanging drop cultures. The medium in which each fragment was embedded consisted of 2 drops of heparinized fowl plasma, one drop of chick embryo extract and one drop of beef embryo extract. Usually 8 cultures were prepared in a petri dish(4). This method permits the study of large numbers of fragments with economy of time and effort. *Roller tube cultures.* The general method has been presented in detail(5). The medium used in these experiments consisted of 9 parts of the Hanks-Simms solution adopted as routine for the suspended cell cultures(1,3) and 1 part of beef embryo extract. Two cc of this mixture was employed in each culture. *pH determinations.* The pH of suspended cell cultures was determined colorimetrically by direct comparison of each culture with a set of standard buffer solutions. The latter were prepared in 25 cc Erlenmeyer flasks each containing 3 cc of solution and the same concentration of phenol red as the Hanks-Simms solution used as culture medium. *Sections of tissue fragments from suspended cell cultures.* At the end of an experiment, Zenker's solution without acetic acid was added to the fragments in each flask. After fixation the fragments from one set of cultures were combined, embedded and stained with hematoxylin and eosin. *Viruses.* The Lansing and Brunhilde strains of poliomyelitis virus have been used(1,2). *Antisera.* Specific monkey antiserum for the Brunhilde strain was obtained through the courtesy of Dr. Jonas Salk. Lansing immune serum was prepared in this laboratory by repeated intramuscular injection of a monkey with infected monkey cord. This serum diluted 1:1000 completely protected mice against 1-3 LD_{50} of virus, but failed to protect in a dilution of 1:5000.

Experimental. Changes in cell morphology

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in fragments from suspended cell cultures. Sections of fragments from 12 serial cultures of the Lansing virus in human embryonic skin-muscle have been examined along with material from 11 successive cultures of another comparable series inoculated with the same virus. Sections from cultures to which no virus was added were, in every case, compared with the corresponding inoculated material. The results were as follows: Eleven cultures included in these 2 series were maintained for periods approximating 30 days (mean period of cultivation 29 days, range 21-32 days). In sections from these, few or no well preserved cells were seen. The changes observed consisted of loss of typical staining properties, nuclear pyknosis and fragmentation of cells. In contrast normal-appearing well-stained cells were present in one or more of the fragments that had not been exposed to the virus. The number of such cells varied but, in all but one instance, were recorded as fairly numerous to numerous. The cells that regularly presented the best state of preservation in uninfected cultures resembled young fibroblasts with large oval nuclei staining pale blue and containing many small chromatin granules. Moreover, the nuclei of muscle cells in many cases appeared to be in good condition. When the period of exposure to the virus was shorter, *i.e.* 16-20 days, differences between inoculated and uninoculated tissue were not always apparent. In certain experiments, however, even by the 16th day of cultivation sections of uninoculated fragments could be easily distinguished from those of infected tissue.

A comparable experiment was carried out with the Brunhilde strain. Sections of embryonic skin-muscle fragments from 10 successive cultures were studied. With the exception of material from the first and seventh culture, cellular degeneration was more marked in the infected fragments. Essentially similar results were recorded in 10 serial cultures of human embryonic intestine inoculated with the Lansing virus.

Examination of material from flask cultures of human embryonic brain inoculated with Lansing and Brunhilde viruses on the whole revealed more extensive cellular degeneration than was noted in control material. The differences, however, in some cases were not so distinct as with the other tissues.

The inhibition of cell migration. Tissue fragments in which certain other viruses have been propagated fail to exhibit migration or peripheral growth of cells characteristic of normal tissues in plasma cultures(4,6,7). In view of these observations and our histologic findings the migratory capacity of cells removed from flask cultures of virus was determined. Plasma hanging drop cultures were prepared with fragments removed from flask cultures at intervals following inoculation of virus. Control cultures were made with uninoculated fragments. Skin-muscle tissue from embryos of not over four months gestation has alone yielded, with regularity, a zone of migrating or growing cells sufficiently dense to provide a reliable indicator system. Other tissues examined were embryonic brain and intestine, as well as foreskin and kidney from young children. The results of a number of experiments were essentially the same. Fragments of skin-muscle from flasks in which virus multiplication was demonstrated by animal inoculation either failed to show cell migration or, if this occurred, it was scanty and within 5 days the emigrating cells exhibited pronounced degenerative changes. In contrast nearly all fragments from control uninoculated flasks developed within 48-72 hours well defined zones of normal-appearing cells which continued to increase and presented a normal appearance for at least 7 days. The effect of the virus on cell migration was not clearly demonstrable until the flask cultures had been maintained for 8 to 21 days.

In Table I are summarized the results of an experiment in which the minimal quantity of virus was determined that exerted a definite inhibitory action on cell migration. Each of a group of 3 flask cultures of human embryonic skin-muscle tissue were inoculated with 0.1 cc of a dilution of the Lansing or Brunhilde strain. After these cultures had been

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TABLE I. Inhibition of Cell Migration in Plasma Explants of Tissues from Flask Cultures Infected with Poliomyelitis Virus.

Virus strain	Dilution of virus inoculated into flask cultures 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	No virus
Lansing*	0/6‡	0/6	1/6	2/6	6/6	10/10
Brunhilde†	0/6	0/6	0/6	5/6	6/6	10/10

* Inoculum: infected mouse brain.

† Inoculum: pooled supernatant fluids from 9th flask subculture in skin-muscle.

‡ $\frac{\text{No. explanted fragments showing normal cell migration after 5 days incubation}}{\text{Total No. fragments explanted from flask cultures}}$

TABLE II. Cytopathogenic Effect of Brunhilde Virus in Roller Tube Cultures of Human Embryonic Skin-Muscle.
Dilutions of Brunhilde virus inoculated.

10^{-2}*	10^{-3}	10^{-4}	10^{-5}	No virus
+ +	+ +	+ +	+ 0	0 0

* 2 cultures were inoculated with each dilution of virus.

+ Culture showing widespread destruction of cells the 5th day after inoculation.

0 = Culture showing normal appearing active cell growth the 5th day.

maintained in the usual manner for 22 days, tissue fragments were removed and plasma hanging drop cultures prepared. From the table it is evident that both viruses, when diluted 10^{-4} and 10^{-3} respectively affected normal cell migration. In the case of the Lansing strain, the pooled fluids removed from the flasks inoculated with 10^{-4} dilution produced paralysis and death in mice whereas those from the cultures inoculated with 10^{-5} dilution caused no disease. With the Brunhilde virus, similar confirmatory experiments in animals have not yet been performed.

The effect of the virus on cell migration revealed by the explant method suggested that the roller tube technic which provides large numbers of actively growing cells might furnish a more convenient and rapid procedure for the demonstration of cell injury. Roller tube cultures of human embryonic skin-muscle were prepared and after 4 days incubation at 37°C were each inoculated with 0.1 cc of a dilution of Brunhilde tissue culture virus. Two roller tube cultures were inoculated with each dilution of virus. Uninoculated cultures were included for comparison. Incubation was continued and the cultures examined daily. The findings on the 5th day after inoculation and which were constant thereafter are summarized in Table II. Lansing tissue culture virus appears to behave in a similar manner in roller tube cultures. These observations indicate that poliomyelitis virus adapted to growth in human tissues rapidly destroys the cells growing out from human skin-muscle.

Effect of virus on tissue metabolism as indicated by decreased acid production. The degenerative changes in the infected cells suggested that cellular metabolism might be impaired. Furthermore, in the case of tissues infected with certain other viruses a diminution in acid production and in their capacity to reduce methylene blue have been described (8,9).

Experiments have shown that acid production in flask cultures of human embryonic brain, intestine and skin-muscle inoculated with Lansing and Brunhilde strains, after a variable interval, declines more rapidly than in control cultures without virus. The time after addition of the virus at which significant differences become apparent has been found to vary according to the amount of virus inoculated and the lot of tissue employed. Moreover, the pH of individual cultures inoculated with the same material may occasionally vary within a fairly wide range. A frequent cause of this variation is failure to insert the stopper tightly. As an example, pH readings recorded in one experiment are given in Table III. Various dilutions of a suspension of mouse brain infected with Lansing virus were inoculated into a series of flask cultures of human embryonic brain. Each figure represents the average of readings of the pH of the medium in three flasks at the end of periods of 3 to 5 days before the addition of fresh medium. By the 15th day the cultures inoculated with the three lowest dilutions exhibited a pH differential of 0.2 to

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9. Plotz, H., *Compt. rend. Soc. Biol.,* 1935, v118, 1400.

0.3 as compared with the controls. We have regarded as significant a difference of 0.2. By the 20th day those inoculated with dilution 10^{-4} likewise showed a significant difference. No differential was observed in the cultures receiving 10^{-5} dilution. Intracerebral injection of mice with the pooled fluids removed on the 15th day showed that virus was present in the cultures inoculated with virus dilutions 10^{-3} and 10^{-4} but not with 10^{-5}.

TABLE III. Determinations of the pH of the Fluid Phase of Flask Cultures of Human Embryonic Brain Inoculated with Lansing Virus.

Dil. of virus† inoculated	Days after inoculation of culture 3	7	11	15	20	24	28
10^{-1}	6.8*	6.8	6.9	7.1	7.2	7.3	7.3
10^{-2}	6.8	6.8	6.9	7.1	7.2	7.3	7.3
10^{-3}	6.8	6.8	6.8	7.0	7.1	7.3	7.3
10^{-4}	6.8	6.8	6.8	6.9	6.9	7.2	7.3
10^{-5}	6.8	6.8	6.8	6.8	6.8	6.8	6.9
No virus	6.8	6.8	6.8	6.8	6.8	6.8	6.9

* Avg of individual pH readings of the medium in three flasks. Supernatant fluids were removed and fresh medium added on the days indicated. Readings were made just before this was done. The pH of the fresh medium varies from about 7.7-7.5.

† The virus consisted of a suspension of infected mouse brain. Three cultures were each inoculated with 0.1 cc of each dilution.

TABLE IV. Specific Inhibition by Immune Serum of the Cytopathogenic Effect of Poliomyelitis Virus as Indicated by Cell Migration.*

Virus inoculum	Type of serum: Lansing immune monkey	Brunhilde immune monkey	Normal monkey
Lansing†	12/12‖	0/12	0/12
Brunhilde‡	0/12	12/12	0/12

* The technic employed was as follows: 0.1 cc of serum inactivated at 56°C for 30 min. was added to each flask culture of human embryonic skin-muscle. One-half hour later 0.1 cc of virus inoculum was added to each flask. The cultures were maintained in the usual manner. Explant preparations were made on the 26th day after inoculation of the flasks.

† Inoculum: undiluted pooled supernatant fluid of 13th subculture of Lansing virus in skin-muscle flask cultures.

‡ Inoculum: undiluted pooled supernatant fluid of 9th subculture of Brunhilde virus in skin-muscle flask cultures.

‖ $\frac{\text{No. explanted fragments showing normal migration after 5 days incubation}}{\text{Total No. fragments explanted from flask cultures}}$

It should be pointed out that the endpoint of viral activity obtained by the explant method as recorded in Table I agrees with that based on pH differential. In general this criterion for the presence of virus in cultures has proved useful and reliable, although in a few instances a significant difference has not been recorded during the period of observation.

Specific inhibition of cytopathogenic effect of virus by immune serum. To obtain additional evidence for the specificity of the degenerative changes noted in infected tissues, the effect of the addition of specific immune serum was determined. Two procedures were employed. In the first, virus and serum were added to flask cultures of skin-muscle and, after incubation, tissue fragments were explanted to plasma drop cultures. In the second, virus and serum were added to freshly prepared roller tube cultures of skin-muscle.

In Table IV the results of an experiment carried out by the first method are presented. It is evident that (a) normal monkey serum fails to protect the cells against the cytopathogenic action of the virus; (b) the antiserum prevents the cytopathogenic effect as indicated by the capacity of the cells to migrate in a normal manner; (c) the effect of the antiserum is type specific.

In this series to which the Lansing strain was added virus was demonstrated by mouse inoculation in the supernatant fluids of cultures containing normal and Brunhilde immune serum but not in those from cultures containing homologous immune serum. Permanent inhibition of viral multiplication by homologous antiserum, however, has not always been observed in this type of experiment.

An experiment in roller tube cultures with Brunhilde virus and antisera against this virus and the Lansing strain has demonstrated a similar capacity of the homologous serum to prevent cell degeneration. Clear cut results were obtained by this method within 5 days after inoculation of the cultures.

Since this inhibitory effect of antiserum appears to be type specific it can be concluded that the degenerative changes observed were produced by the action of the virus on the cells.

Summary and discussion. The experiments which have been described demonstrate the capacity of the Lansing and Brunhilde strains of poliomyelitis virus to cause cell injury and death. This cytopathogenic property is revealed (1) by degenerative changes produced in infected tissue fragments in flask cultures and apparent on histologic examination; (2) by failure of such tissue fragments to exhibit normal cell migration when explanted to plasma cultures; (3) by degeneration of newly emigrated cells in roller-tube cultures; (4) by decreased acid production by infected cells. The conclusion that certain of these manifestations of injury are induced as a result of infection by the virus is further supported by the fact that type specific immune serum prevents their development. These phenomena are of interest from two general points of view. First, they leave no doubt that poliomyelitis virus *in vitro* can multiply in cells other than those of the nervous system and cause profound injury of such cells. Secondly, they provide criteria by which the presence of the virus can be recognized *in vitro* and hence may afford a basis of technics for isolating virus from patients or animals, for the quantitative assay of virus, for serologic typing and possibly for the screening of chemotherapeutic and antibiotic substances. Further study will be required of the reliability and practicability of the application of these phenomena to such ends.[†]

[†] The authors are grateful to Marguerite Buckingham, Gloria Florentino, Carolyn Keane, and Sheila Richardson for essential technical assistance and to Dr. Duncan Reid and the members of the staff of the Boston Lying-in Hospital for their assistance in obtaining materials for this study.

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Biological Studies on *Entamoeba histolytica*. IV. Direct Action of the Antibiotic, Prodigiosin.[*] (18203)

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Antibiotics have been applied against *Entamoeba histolytica* as part of the search for more effective chemotherapeutic agents. Although various accepted antibiotics (penicillin, streptomycin, aureomycin, chloromycetin, etc.) have been tested *in vitro,* no clear-cut evidence has been presented of their direct action upon the amoebae. A recent report from Anderson's laboratory aptly summarizes the situation: "All antibiotics tested to date have shown some degree of bacteriostatic activity, and death of the ameba in cotton-stoppered tubes is shown to be due to inhibition of growth of the associated bacteria rather than to direct action of the antibiotic on *E. histolytica*"(1). The present experiments were designed to meet these objections, and the data presented below are submitted as the first proof of direct action by an antibiotic on *E. histolytica.*

Our original interest in prodigiosin sprang both from the long-known antagonism exhibited by chromogenic strains of *Serratia marcescens* against various microorganisms, and the feasibility of using the corresponding antibiotic pigment, prodigiosin, whose extraction and structure had been elucidated by Wrede and co-workers(2). Antibacterial activity has been demonstrated against gram-positive types, including *Bacillus anthracis* (3), *Corynebacterium diphtheriae* and *Neisseria gonorrhoeae*(4), *Pasteurella pestis*(5),

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[*] Part of a project supported by a research grant from the National Institutes of Health, Public Health Service.

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B. anthracis and staphylococci(6). Hettche claimed that *B. anthracis* was immediately killed by 1:50,000 prodigiosin, one-half the concentration required for staphylococci, while gram-negative "colon and paratyphoid" bacilli were unaffected. Free-living protozoa have been reported susceptible to chromogenic strains of *S. marcescens: Glaucoma, Colpoda, Paramecium*(7,8), and soil amoebae (9). Fischl(10) demonstrated that prodigiosin perchlorate was trypanocidal in mice infected with *T. brucei.* The present authors (11) presented their partial data before the American Society of Parasitologists. The only report of fungicidal activity is that of Lack (12), who found that *Coccidioides immitis* was destroyed *in vitro* after 48 hours' exposure to 1:100,000 prodigiosin.

Materials and methods. Prodigiosin was extracted in this laboratory from surface cultures of *S. marcescens* grown on Hayes' medium(13) adjusted to pH 6.7 and containing 1.5% agar. The cultures were harvested after four days' incubation in the dark at 23-25°C, and the pigment was extracted according to the method of Wrede and Hettche(14). The properties of our compound agreed with those of the original, which has been identified as a tripyrrole methane bearing the formula $C_{20}H_{25}ON_3$. As a further check the absorption characteristics were measured with a Beckman quartz spectrophotometer, model DU.† The curves (Fig. 1) conformed strikingly to those of Ehrismann and Noethling (15), although there was an apparent typographical error in the value accorded the ordinate in their paper.

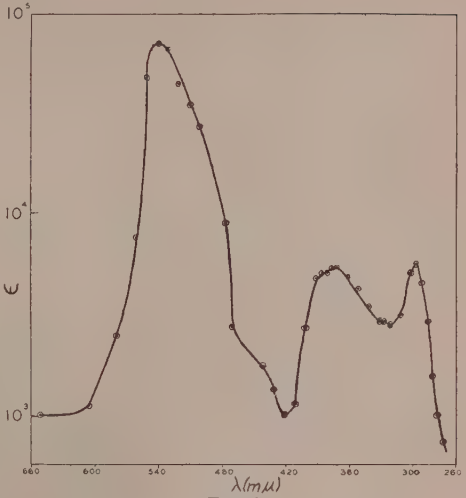

FIG. 1.

Absorption curve of prodigiosin. Extinction coefficient (ε) plotted against wavelength (mμ).

Two distinct strains of *E. histolytica* were employed: the *UC* strain associated with a mixed bacterial flora, and the *NRS* strain associated only with *Aerobacter aerogenes.* The test medium was the same buffered egg-yolk infusion (pH 7.40) plus rice starch in which stocks have been maintained for several years(16). In different experiments culture tubes contained either 10 ml or 30 ml medium, the former receiving 0.5 ml inoculum, the latter 1.0 ml. Bacterial populations were measured by decimal-dilution plating, counts being made of the last two dilutions. Amoebic inocula were counted on hemocytometers and exceeded 100,000 in all critical experiments; yields were usually recorded after 48-72 hours as simply present or absent. In all apparently negative tubes, bacterial counts were made and 0.2 ml sediment

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12. Lack, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 656.

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14. Wrede, F., and Hettche, O., *Ber. Deutsch. Chem. Ges.*, 1929, v62, 2678.

† These measurements were made under the direction of Dr. I. M. Klotz of the Department of Chemistry.

15. Ehrismann, O., and Noethling, W., *Biochem. Z.*, 1936, v284, 376.

16. Balamuth, W., *Am. J. Clin. Path.*, 1946, v16, 380.

TABLE I. Effects of 48-hr Exposure to Prodigiosin in Monobacterial Cultures of *Entamoeba histolytica* (*NRS* strain with *Aerobacter aerogenes.*)

	Concentrations of prodigiosin					
Features observed	Control	1:2 × 10^6	1:10^6	1:8 × 10^5	1:4 × 10^5	1:2 × 10^5
Survival of amoebae*	+	+	+	+	—	—
Bacteria (× 10^6 per ml)	780	950				1100
Oxidation-reduction potential (E_h in mv.)	—185	—185	—220		—240	—210
pH	7.08	7.10	7.03	7.05	7.04	7.20

* The same amoebacidal endpoint was obtained with inocula varying between 17,000 and 251,000 amoebae.

TABLE II. Effects of 48-hr Exposure to Prodigiosin in Cultures of *Entamoeba histolytica* (*UC* strain with Mixed Flora.)

		Concentrations of prodigiosin				
Features observed	Inocula	Control	1:2 × 10^5		1:10^5	
Survival of amoebae	102,000	+	—		—	
	391,000			+		—
Bacteria (× 10^6 per ml)	7.3	445	351		213	
	27.9			307		264
Oxidation-reduction potential (E_h in mv.)		—280	—217		—186	
				—208		—222
pH		6.99	7.14		7.15	
				7.09		7.12

was subcultured into sterile medium for an additional 72 hours' incubation. Oxidation-reduction potentials (E_h) and pH were measured with a model G Beckman meter; for the former, duplicate gold-plated platinum electrodes were inserted permanently in some series of tubes. Stock solutions of prodigiosin were maintained in ethanol and checked for sterility before use. Dilutions were prepared in the range 1:20,000,000 to 1:100,000, and controls received an equivalent concentration of ethanol (max. 0.75%).

Results. Tables I and II summarize for the two strains respectively the major effects on viability of amoebae and associated bacteria, as well as changes in E_h and pH. Amoebae in the monobacterial culture proved more susceptible to the action of prodigiosin, even in the absence of demonstrable action against *A. aerogenes.*‡ Owing to more abundant growth of stocks of the *UC* strain, this culture was used to study the influence of size of the amoebic inoculum. As Table II

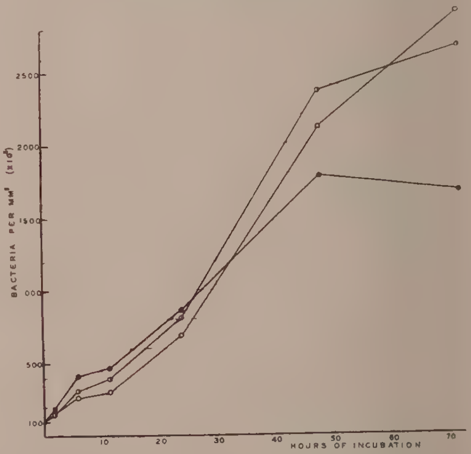

FIG. 2.

The effects of ethanol and prodigiosin on the growth of *Aerobacter aerogenes.*

● regular test medium.

Half solid right circle—0.75% ethanol added to test medium.

○ 0.75% ethanol + 1:100,000 prodigiosin added to test medium.

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‡ This same relative reaction has been observed in this laboratory in the case of recognized amoebacidal agents. The implications for drug-testing procedures will be discussed elsewhere.

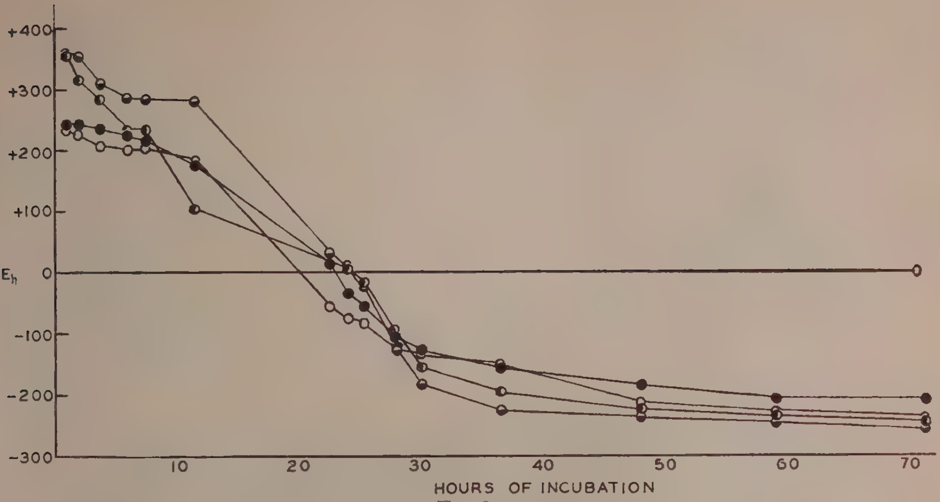

Fig. 3.

Changes in oxidation-reduction potential (E_h) of monobacterial cultures of *Entamoeba histolytica* (NRS strain with *Aerobacter aerogenes*), in different concentrations of prodigiosin.

● Control
Solid lower half circle—1:400,000
Solid left half circle—1:1,000,000
○ 1:200,000

shows, relatively large amoebic inocula resulted in greater resistance to prodigiosin at 1:200,000, and this could not be attributed to the bacteria. No survival was recorded for either combination at 1:100,000, regardless of size of inoculum, and we regard this as the lowest amoebacidal concentration of prodigiosin under our experimental conditions. Effects were clearly discernible against individual amoebae at 1:2,000,000; they became highly vacuolated and motionless, and acquired a pinkish tint. At higher concentrations the amoebae were lysed rapidly, so that even after 12 hours few carcasses persisted.

The effects on bacteria are seen most clearly in monobacterial cultures. There was no evidence of inhibition of *A. aerogenes* either by prodigiosin or ethanol in the concentrations employed (Fig. 2). Also in the case of the *UC* mixed flora, large populations of viable bacteria were present even at the highest concentrations of prodigiosin.§

The maintenance of low (*i.e.*, negative) oxidation-reduction potentials is a prime requisite for viability and growth of intestinal amoebae(17,18). Bradin and Hansen(19) report E_h values of approximately –200 mv for their control cultures. Since the redox potential of cultures is controlled chiefly by the bacterial flora(18), it would be expected from the present study that control and experimental cultures should agree quite closely in this regard. Fig. 3 and 4 disclose this to be true, and effectively rules out the possibility that amoebacidal action can be attributed to unfavorable potentials. It may be noted that the potentials fell more slowly in the monobacterial cultures, requiring some

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§ Dr. E. Hansen has kindly tested a sample of our prodigiosin against three monobacterial cultures of *E. histolytica,* involving respectively organism *t*, *Clostridium perfringens,* and the streptobacillus of Shaffer and Frye. Dr. Hansen concluded (in litt.) that "Prodigiosin, in alcoholic solution, is amebacidal at 1:100,000, the alcohol present (0.5% ethanol) contributing to the toxic action," especially through marked inhibition of the bacterial associates. Our most complete bacterial analyses were conducted after receipt of this report. The flora in our cultures clearly did not respond in the same way as above, but rather in the manner reported by Hettche for members of the gram-negative intestinal flora.

17. Jacobs, L., *J. Parasit.*, 1941, v27 (Suppl.), 31.
18. Chang, S., *Parasit.*, 1946, v37, 101.
19. Bradin, J., and Hansen, E., *Am. J. Trop. Med.*, 1950, v30, 27.

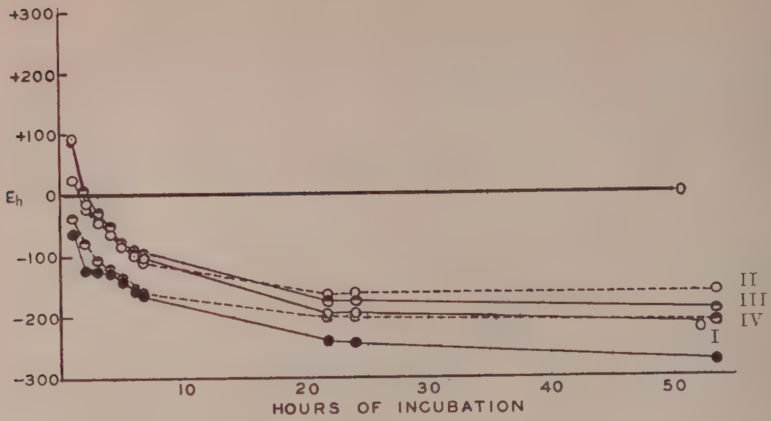

Fig. 4.

Changes in oxidation-reduction potential (E_h) of cultures of *Entamoeba histolytica* (*UC* strain with mixed flora) in different concentrations of prodigiosin, with different amoebic inocula.

● Control.
I—1:200,000 (102,000 amoebae)
II—1:200,000 (391,000 amoebae)
III—1:100,000 (102,000 amoebae)
IV—1:100,000 (391,000 amoebae)

30 hours to reach –200 mv in comparison to 10 hours for the *UC* strain, but in all cases controls and experimentals behaved similarly. The pH of all cultures was held in the range pH 6.8 to 7.2 through the use of well-buffered media.

Summary and conclusions. (1) Prodigiosin was employed in the range 1:20,000,000 to 1:100,000 against two distinct strains of *E. histolytica.*

(2) The monobacterial culture (*NRS* strain grown with *A. aerogenes*) proved more susceptible, failing to survive at 1:400,000. The mixed culture (*UC* strain) failed to survive at 1:100,000, even with relatively huge inocula of amoebae.

(3) Analysis disclosed that the bacterial flora, oxidation-reduction potentials and pH were not adversely affected by the experimental treatment.

(4) The general conclusion is reached that prodigiosin exerts a profound, direct action against *E. histolytica in vitro.* This is apparently the first record of proved direct action of an antibiotic against *E. histolytica.*

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Further Studies on the Thermostable Leukocytosis Factor of Exudates. (18204)

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The rise in the number of circulating leukocytes with inflammation is referable to at least two factors released by cells injured at the site of injury: (1) a thermolabile leukocytosis-promoting factor (the so-called LPF) and (2) a thermostable leukocytosis factor which is closely associated with the pyrogenic factor, pyrexin, recovered particularly from acid exudates(1-3).

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* Aided in part by a grant from the National Advisory Cancer Council, U. S. Public Health Service. This is No. 42 of the series "Studies on Inflammation."

1. Menkin, V., *Am. J. Path.*, 1940, v16, 13.

An attempt has been made to purify further the thermostable leukocytosis factor. The latter has been recently recovered in a crystalline form. This has been obtained by freeing it from pyrexin, so that these crystals induce only a leukocytosis without any pyrogenic or leukopenic(4) properties. The crystals, the extraction of which is to be presently described, are thermostable in nature. They thus seem to reproduce all the effects of the original and crude thermostable component described(3).

Method. An active fraction of canine pyrexin from exudates is weighed in varying amounts, usually around 20 mg. The powder is suspended in about 20 cc of distilled water. About 20 cc of saturated $(NH_4)_2SO_4$ is added, so that the pyrogenic factor is consequently subjected to the salt at one-half saturation. The entire mixture is then dialyzed in a cellophane tube against tap water. About 24 hours later when the material is free of sulfate ions, the mixture is thoroughly centrifuged. The supernatant phase is a crude material which contains 3 components, namely the pyrogenic, the leukopenic, and the thermostable leukocytosis factors. The precipitate fraction contains considerable pyrogenic activity, when injected intravenously into rabbits. The crude supernatant, containing the 3 components listed, is now diluted with an equal volume of distilled water. About 10 cc portions are poured into a test tube which in turn is sealed. The tube is then refluxed in boiling water for a period ranging from 17 to 20 hours. After that time, the tube is cooled either at room temperature or preferably in a refrigerator. Crystal formations then occur, which by gross examination appear as small needles, but microscopically usually resemble irregularly-shaped crystalline flakes. The contents of the tube are centrifuged, and the crystals are collected as a precipitate. The crystals are injected into the circulation of dogs and periodic white counts are made for about 6 to 24 hours.

TABLE I. Effect of Crude Material on Leukocyte Count in the Blood.

Dog No.	Basal white count, per cubic mm	White count about 1 hr after inj. of material, per cubic mm	Highest white count within about 1 day, per cubic mm	Max. rise in temp., °F
54-T	11150	4000	16350	1.2
156-T	4675	1500	9200	2.7
63-T	9550	3950	19950	2.7
98-T	15500	12700	23800	1.0
63-T*	10950	4750	20100	1.6
157-T	11550	3150	20500	1.9
Avg	10563	5008	18317	1.9

* 5-year-old pyrexin utilized.

TABLE II. Effect of Crystals of Thermostable Leukocytosis Factor on Leukocytes of the Blood.

Dog No.	Basal white blood cell count, per cubic mm	White blood cell level about 1 hr after inj. of crystals, per cubic mm	Highest count within about 24 hr after inj. of crystals, per cubic mm	Highest change in temp., °F
129-T	14800	15300	25400	−0.4
156-T	3100	5450	7550	+1.6
54-T	13550	13400	19450	+0.2
63-T	9875	13300	16425	+0.2
156-T	8875	13750	26800	+1.2
129-T*	18650	15500	23850	+0.7
98-T	13850	13750	19250	+0.4
97-T†	9200	9150	18950	0
Avg	11488	12450	19709	0.5

* 5-year-old pyrexin utilized to oztain the crystals of the thermostable leukocytosis factor.

† Filtered the crystals, and then took the wet filter paper and washed off the crystals in distilled water.

Results. When pyrexin is treated at half saturation with ammonium sulfate and dialyzed, as indicated in the scheme of extraction, a so-called crude material is obtained in which some amorphous material is seen to be suspended. This fraction is thoroughly centrifuged. The precipitate or sediment appears to contain considerable pyrogenic activity. When suspended in a minimal amount of aqueous fluid and injected intravenously into rabbits, the latter develop a rapid rise

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2. Menkin, V., *Arch. Path.*, 1940, v30, 363.
3. Menkin, V., *Blood, The Jour. of Hematol.*, 1949, v4, 1323.
4. Menkin, V., *Arch. Path.*, 1946, v41, 50.

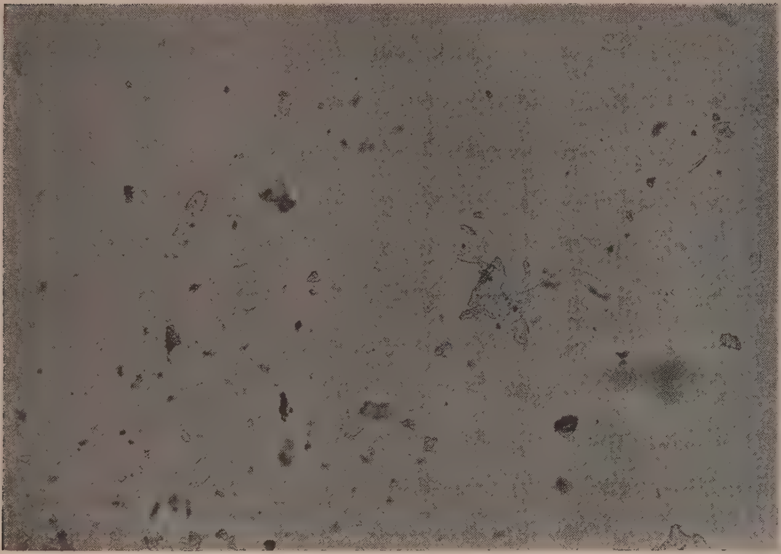

FIG. 1.

One-day-old crystals of the thermostable leukocytosis factor. These crystals were derived from a sample of pyrexin which had been isolated 5 years previously. 400 gamma of these crystals induced a rise of 77.5% in the 1-lobe form of granulocytes. ×134.

in rectal temperature. The average rise is 2.7°F. This effect in rabbits has already been described(5).

The supernatant phase after centrifugation of the precipitate, as described above, upon injection intravascularly into dogs yields at first a leukopenia subsequently followed by a leukocytosis. Furthermore, there is a rise in rectal temperature which averages 1.9°F (Table I). These results are similar to the ones described with the thermostable component of the leukocytosis factor(3). The activity of such pyrexin preparations may be found even 5 years after extracting the pyrogenic factor from inflammatory exudates (dog No. 63-T, Table I).

Following further the scheme of extraction of the thermostable leukocytosis factor as outlined above, a crystalline fraction can be obtained which has neither any initial leukopenic effect nor has it any pyrogenic capacity. These crystals, however, induce in several to 24 hours an appreciable state of leukocytosis. One hour after administration of the crude supernatant material there is a fall in the leukocyte level amounting to 53% (Table I). On the contrary when the crystalline material is injected there is no initial leukopenia, but there is even a slight rise in the count averaging 8% (Table II). Nevertheless, these crystals induce a leukocytosis similar to the crude material averaging 72% (Table II). The leukocytosis induced by the crude supernatant fraction is of the same order, namely 73% (Table I). Thus, crystals of the thermostable leukocytosis factor which are heat stable and which have neither any initial leukopenic or pyrogenic effects (0.5°F, Table II) have been obtained by the above scheme of extraction.

An illustration of these crystals appears in Fig. 1. The crystals which in the gross resemble needle-like projections, microscopically appear as irregularly-shaped, doubly refractile crystals (Fig. 1). The crystals in contrast to the thermolabile LPF(6) induce no change in the amino nitrogen after hydrolysis. This would suggest that the mater-

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5. Menkin, V., *Arch. Path.*, 1945, v39, 28.

6. Menkin, V., *Blood, J. Hematol.*, 1948, v3, 939.

ial is not polypeptide in character. The total nitrogen also yields a figure, namely 2.1%, which is lower than the active polypeptide of the thermolabile LPF. The latter has been found to have 12.3% nitrogen.

The Molisch test for carbohydrates is on some samples of the crystals very strongly positive; whereas in other samples especially if the crystals are dried previously, it is quite weak or absent. The correlation of biological activity and the Molisch reaction has as yet not been studied. The variability in the intensity of this test merely suggests that perhaps the active non-desiccated crystals of the thermostable leukocytosis factor may have a carbohydrate linked to the structure of the crystals. Further studies will establish this point on a firmer basis.

A study was undertaken to determine whether the crystals of the thermostable leukocytosis factor induce a discharge into the circulation of immature or 1-lobe form of granulocytes. At the time that the crude thermostable factor was identified as associated with pyrexin, it was also established that this material causes a discharge of immature granulocytes into the blood stream(3). A similar study was carried out with the crystals of the thermostable leukocytosis factor. The data indicate clearly that the percentage of 1-lobe form of granulocytes augments as early as one hour after administration of either the crude material or the crystals of the thermostable leukocytosis factor. Within about one hour, there is an average increase which almost is double that of the basal level, namely 18 to 35%.

When the crystals are either washed repeatedly with distilled water or when they are desiccated, they lose some of their activity. The absolute white count is barely increased. Nevertheless, the differential microscopic picture indicates a rise in the percentage of 1-lobe form of granulocytes. There is also a marked increase in the absolute number of 1-lobe granulocytes. As pointed out previously, it seems as if a study of the differential leukocytic formula is a more delicate test than the absolute white count in determining the effectiveness of a given material(3,7). On the other hand, when either the distilled water utilized to wash the crystals, or the supernatant fluid alone but freed of suspended thermostable crystals are injected in dogs, there is no appreciable change in either the absolute white count level or in the differential formula. These facts point further to the capacity of the crystals in increasing the number of leukocytes in the blood stream.†

Discussion and conclusions. The significance of the thermostable leukocytosis factor as a contributing factor in the mechanism of leukocytosis with inflammation has already been pointed out(3). At the time no conclusion could be reached as to whether the thermolabile LPF and the thermostable leukocytosis factor are one and the same substance, or whether the two factors are distinct entities. The present studies bearing on the nitrogen content of the crystals of the thermostable leukocytosis factor support the view that the two factors are two distinct substances.

In conclusion, the thermostable leukocytosis factor, recovered especially from acid exudates, and which is found in association with the pyrogenic factor, pyrexin, has been crystallized, and the crystals as such have essentially neither pyrogenic nor leukopenic properties. The crystals of the thermostable leukocytosis factor induce a leukocytosis characterized by a discharge into the circulation of immature 1-lobe form of granulocytes.

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7. Menkin, V., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 448.

† As a further control, several dogs received an intravascular injection of a similar crystalline material derived from a toxic euglobulin derived from exudates and termed necrosin(5). These crystals were in all cases incapable of inducing a leukocytosis in the animals (unpublished studies). These latter studies point to the specificity of the thermostable crystals described in the present communication in inducing a state of leukocytosis.

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Intraperitoneal Injections of India Ink and Ox Bile and the Blood Lactic Acid in Rats.* (18205)

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We have shown(1,2) that the intraperitoneal injection of certain particulate and large molecular substances lowers the body-temperature in white rats, and that this fall in body-temperature seems to be associated with the removal of these substances from the peritoneal cavity by the reticulo-endothelial system. Further, it appears that the fall in body-temperature can occur without shock as shown by the small coincident hematocrit and plasma protein changes(2). However, it seemed advisable to investigate further the possible role of shock in the production of hypothermia following the intraperitoneal injection of these substances. For this purpose we compared the changes in blood lactic acid after intraperitoneal injection of India ink (a representative of the particulate substances, which cause hypothermia) with the changes following intraperitoneal injection of ox bile, which is known to produce shock(2).

Experimental. The methods of injection, the technic of measurement of body-temperature, and the care of the animals have been described previously(1). Young adult white rats were used in all instances. Higgins India Ink was used as purchased. Ox bile was used as received from the slaughter house. Blood lactic acid was determined by the method of Barker and Summerson(3) as modified by LePage(4). The rats were kept in individual wire cages at room temperature (25°-27°C) throughout the experiment. Except as indicated the experiments were performed with the rats under pentothal-sodium anesthesia in order to avoid the possibility that any observed rise in blood lactic acid might have been caused by excitement. Preliminary procedures were carried out in the following order:

High colonic temperatures were measured, a sample of blood for lactic acid analysis was taken by clipping the tip of the tail, and, whenever pentothal sodium was used, it was injected subcutaneously immediately thereafter. Twenty minutes later, in order, the temperature was taken again, another sample of blood was collected, and the experimental substance, India ink or ox bile, was injected intraperitoneally. The rats were then placed in individual wire cages. Body-temperatures were measured and blood-samples were drawn for lactic-acid determination at intervals throughout the experiment.

Results. In all of the figures, the numbers of the individual rats are shown at the end of the curves so that each lactic-acid curve can be compared with its corresponding body-temperature curve.

Fig. 1 shows the results of intraperitoneal injection of 2 cc of India ink without anaesthesia. With these rats, as in the other experiments (Fig. 2, 3, 4) there is a strong tendency to high blood lactic acids in the first sample. Presumably this is a reaction to the excitement of the first handling in the morning although the excitement was not otherwise apparent. It is not repeated later even in the first experiment (Fig. 1) in which no anaesthesia was used.

Fig. 2 shows the changes in body-temperature and in blood lactic acid following the

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* Supported under contract with the Office of Naval Research.

1. Hill, R. M., and Rutledge, E. K., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 9.

2. Hill, R. M., and Rutledge, E. K., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 310.

3. Barker, J. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.

4. LePage, G. A., Method for Lactic Acid, p. 104, Manometric Techniques and Related Methods for the Study of Tissue Metabolism, by W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Burgess Publishing Co., Minneapolis, 1948.

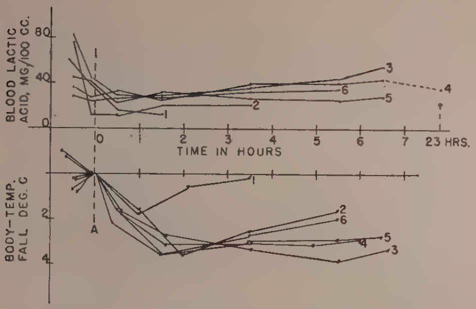

Fig. 1.
Fall in body temperature and blood lactic acid curves after intraperitoneal injection of 2 cc of India ink without anesthesia. Rat numbers given at the ends of the curves.

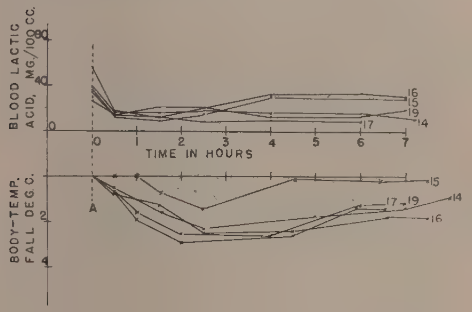

Fig. 2.
Body temperature and blood lactic acid curves following the subcutaneous injection of 0.2 cc of 2.5% pentothal sodium per 100 g body weight, without any other treatment. Rat numbers are given at the ends of the curves.

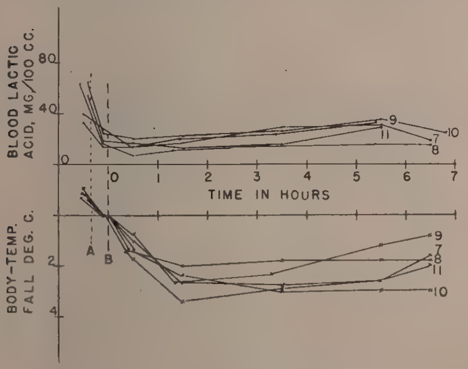

Fig. 3.
Body temperature and blood lactic acid curves after the subcutaneous injection of 0.2 cc of 2.5% pentothal sodium per 100 g body weight at A, and the intraperitoneal injection of 2 cc of India ink at B. Rat numbers are given at the ends of the curves.

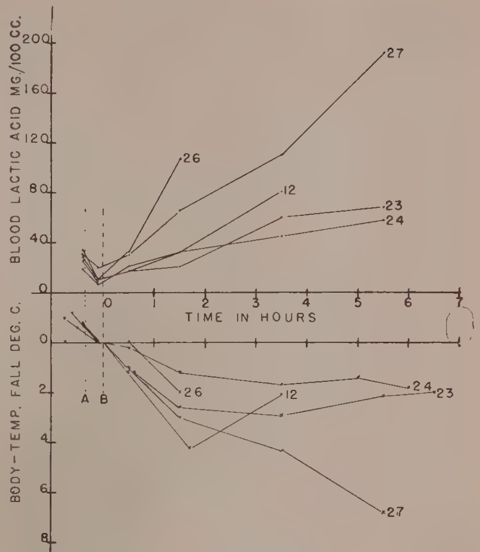

Fig. 4.
Body temperature and blood lactic acid curves following the subcutaneous injection of 0.2 cc of 2.5% pentothal sodium per 100 g body weight at A, and the intraperitoneal injection of 0.5 cc of ox bile at B. Rat numbers are given at the ends of the curves.

subcutaneous injection of 0.2 cc of 2.5% pentothal sodium per 100 g body-weight, without any other treatment. Pentothal sodium used in this way causes a hypothermia comparable to that following the intraperitoneal injection of India ink, and like India ink produces no significant change in the blood lactic acid.

Fig. 3 shows the changes in body-temperature and in blood lactic acid subsequent to subcutaneous injection of pentothal sodium followed in 20 minutes by the intraperitoneal injection of 2 cc of India ink. The changes produced in body-temperature and in blood lactic acid are not significantly different from those after injection of pentothal sodium or India ink alone (Fig. 1 and 2). It is noticeable that there is no additive effect of the two substances on body-temperatures when they are both injected.

Fig. 4 shows the changes in body-temperature and blood lactic acid after subcutaneous injection of pentothal sodium followed by intraperitoneal injection of 0.5 cc of ox bile. With the exception of Rat 27, the falls in body-temperature were comparable to those in the other experiments. Ox bile injected intraperitoneally is extremely irritating. Rat 27 was in a moribund condition when the last body-temperature was taken and died shortly thereafter. Blood lactic-acid values for all the rats receiving ox bile rose sharply and continued to rise for the duration of the experiment.

Discussion. The body-temperature and blood lactic-acid curves following the intraperitoneal injection of India ink (Fig. 1), of pentothal sodium (Fgi. 2) and of India ink plus pentothal sodium (Fig. 3) are much alike. There is no evidence that the treatment caused increases in blood lactic acid. The initial high values for lactic acid found in most of the rats were surprising, particularly in view of the fact that the excitement, which was the probable cause of these high values, was not otherwise apparent in the behavior of the animals. The second value taken 25 minutes later was in some cases less than half of the initial value and must be taken as the "normal" value for the individual rat. There is little evidence from the lactic-acid curves that the subsequent hourly handling and bleeding produced comparable excitement. All the rats had been handled frequently and were gentle. This experience emphasizes the care with which "initial" values in experiments on rats must be judged.

Following the intraperitoneal injection of 0.5 cc of ox bile plus pentothal sodium, the response is different. There is a greater variation in the degree of hypothermia, a variation that seemed to reflect the physical condition of the animal. Rat 26 died at the end of 2 hours. In all instances there was a pronounced increase in the blood lactic acid.

We believe that these experiments give further support to our belief that the fall in body-temperature following the intraperitoneal injection of particulate substances is not a reflection of shock.

Summary. Adult white rats were treated in 4 ways. In 3 of these, (a) intraperitoneal injection of 2 cc of India ink, (b) subcutaneous injection of 0.2 cc pentothal sodium per 100 g, and (c) treatment (b) followed in 20 minutes by treatment (a), blood lactic acid remained normal but body-temperature fell. In the fourth treatment, subcutaneous injection of pentothal sodium followed in 20 minutes by intraperitoneal injection of 0.5 cc ox bile, a similar fall in body-temperature occurred which was accompanied by a marked rise in blood lactic acid. The rise in blood lactic acid is taken as an indication of shock.

These experiments indicate that the hypothermia produced by the intraperitoneal injection of India ink is not secondary to shock.

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Observation of a Spreading Factor Present in Mammary Gland Extracts.*† (18206)

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It is well known that at the onset of pregnancy a phase of rapid growth of the lobule-alveolar system of the mammary gland is initiated. At this time there is not only an extension of the major ducts but also a rapid proliferation of side buds which invades the adjacent subcutaneous fatty connective tissue (fatty pad). In order to facilitate the forward growth of the end buds, it seemed possible that a mucolytic enzyme or collagenase might be elaborated by the growing mammary gland cells which, through liquefication

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[*] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1223.

[†] The hyaluronidate and hyaluronidase used in this investigation were generously provided by the Schering Corporation, Bloomfield, N. J.

of the adjacent connective tissue, would make possible the penetration of these growing buds. Several spreading factors, including hyaluronidase, have been found to act in a manner similar to the one described(1-4).

The present investigation was initiated to explore the possibility of finding such an enzyme or factor.

Methods and materials. Both male and female adult albino rats weighing from 150 to 250 g were used. In removing the mammary glands, the animals were skinned, leaving the glands attached to the skin. The blood was squeezed out, then the fatty pads containing the mammary glands were carefully dissected excluding all lymph nodes and other tissue. The stage of pregnancy of each female was determined by examining the size of the fetuses. Since at times it was impossible to extract the glands immediately, some were held at –15°C until this could be done. Twenty-five ml of 0.1 M sodium acetate buffer pH 6.0 with 0.15 M sodium chloride was added to the glands and the material homogenized in a Waring blendor. The homogenate was then centrifuged for 20 minutes at 2,000 r.p.m. The milky supernatant fluid was then pipetted off, avoiding as far as possible the inclusion of any fraction of the fat layer which formed on top.

A test for spreading factors was first tried. This involved the injection of 0.2 ml of the test material and 0.1 ml of 2% T-1824 (Evans Blue) intradermally into a shaved white rabbit. The dye served as an indication of the extent of fluid spread. This test is only a slight modification of several suggested for the assay of hyaluronidase(5,6) The test material in most cases was the mammary gland extract diluted 10×; however, testicular extract diluted 10× and the acetate sodium chloride pH 6 buffer were used as controls. The injections produced blebs on the rabbits' skin which were measured immediately after injection and which were uniformly circular. After 30 minutes the length and width of the blebs were measured again. The net area was then determined.

A turbidimetric method of assay was also employed(7). Several preliminary assays conducted with purified hyaluronidase indicated the reliability of potency of the enzyme preparation and the reproducibility of the method. Before the hyaluronidase assays of the mammary gland preparations could be made, they had to be extracted with either acetone, alcohol or ether to remove the lipids and to precipitate the biologically active material. The precipitate was suspended in 25 ml of the acetate sodium chloride pH 6 buffer, and after stirring 10 minutes the suspension was filtered through a No. 1 filter paper. These filtrates were then assayed.

Experimental. Initial spreading type tests on mammary gland extracts from 5 castrate female rats and 5 females in normal estrous cycles failed to give any indication of the presence of a spreading factor; however, the mammary gland extracts from females pregnant 6 to 11 days produced definite spreading on the rabbits' skin. Several testicular extracts used as the positive controls gave comparable spreading effects, while the acetate sodium chloride pH 6 buffer injections which did not spread were the negative controls. As the initial tests on the extract of mammary glands from pregnant females indicated a spreading factor, it was decided to try the turbidimetric method of assay for hyaluronidase in order to determine if the spreading effect was due to this enzyme. Eight turbidimetric assays on a sample of purified hyaluronidase which gave positive results served as a check. Ten mammary gland extracts from pregnant female rats which gave a spreading effect on rabbits' skin‡ were turbidimetrically assayed. In no

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2. Burnet, F. M., and Stone, J. D., *Australian J. Exp. Biol. Sci.*, 1947, v25, 219.

3. Burnet, F. M., and Stone, J. D., *Australian J. Exp. Biol. Sci.*, 1948, v26, 71.

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5. Bacharach, A. L., Chance, M. R. A., and Middleton, T. R., *Biochem. J.*, 1940, v34, 1464.

6. McClean, D., and Hale, C. W., *Biochem. J.*, 1941, v35, 159.

7. Tolksdorf, S., McCready, B. A., McCullagh, D. R., and Schwenk, E., *J. Lab. Clin. Med.*, 1949, v34, 74.

TABLE I. Spreading Factor Present in Rat Mammary Gland Extracts.

	Spreading type assay			
Type of extract	No. of animals	Initial bleb, mm	Final bleb, mm	Area of spread, sq mm
pH 6 buffer	14	14×14	15×15	22.8
Testis extr.	17	14×14	24×26	336.0
Castrate ♂	5	14×14	15×15	22.8
,, ♀	5	13×14	14×14	11.0
Normal ♂	5	13×13	14×14	21.2
,, ♀	5	13×14	15×15	33.8
1 day pregnant	3	14×14	15×15	22.8
2 days pregnant	2	13×13	14×15	32.2
3 ,, ,,	4	14×14	16×18	72.3
4 ,, ,,	3	14×14	18×18	100.6
5 ,, ,,	4	13×13	19×20	166.6
6 ,, ,,	4	14×14	20×20	160.3
7 ,, ,,	4	14×14	20×20	160.3
8 ,, ,,	3	14×14	20×21	176.0
9 ,, ,,	3	13×14	20×21	187.0
10 ,, ,,	5	14×14	21×21	199.7
11 ,, ,,	3	13×13	21×22	231.0
12 ,, ,,	4	14×14	23×24	279.7
13 ,, ,,	3	14×14	22×23	243.6
14 ,, ,,	3	14×14	22×22	226.3
15 ,, ,,	3	13×14	20×21	187.0
16 ,, ,,	4	14×14	20×20	160.3
17 ,, ,,	2	14×14	19×19	128.9
18 ,, ,,	3	14×14	16×18	72.3
19 ,, ,,	3	13×13	14×15	32.2
20 ,, ,,	2	14×14	15×15	22.8
21 ,, ,,	2	13×14	14×15	22.0

case did these mammary gland extracts give any indication of hydrolyzing the substrate hyaluronidate (Table I).

Since the results of the assays on the mammary gland extracts indicated that the spreading factor was not hyaluronidase, all further assays employed the spreading factor technic. Mammary gland extracts from 5 normal male rats and 5 castrate males gave no indication of a spreading factor.

Assays of mammary gland extracts from 67 female rats taken during various stages of pregnancy indicated the presence of measurable amounts of spreading factor except during the first and last 2 days of pregnancy. The amounts of spreading factor extracted from the mammary gland increased gradually with each day of pregnancy until the maximum was reached on about the twelfth day following which there was a gradual decline in the level of the spreading factor during the remainder of pregnancy.

It was next deemed necessary that the enzymatic character of the spreading factor be checked. Tests using the spreading method of assay were made to determine the inactivation temperature of the mammary gland spreading factor. Testicular spreading factors were run simultaneously.

Mammary gland extracts from 5 pregnant females and 5 testicular extracts were heated to 55°C in a water bath for 10 minutes. None of the extracts were inactivated by these conditions. Five different mammary extracts from pregnant rats and 5 more testicular extracts were heated to 60°C in a water bath for 10 minutes. The testicular extracts gave no spreading effect while the mammary extracts were still active. Tests of a number of mammary gland and testicular extracts which had been heated to 70°C and higher showed complete inactivation.

Discussion. In view of the experimental data it seems rather certain that increased amounts of a spreading factor are present in mammary gland extracts from pregnant female rats, while the glands of normal male and female rats, as well as castrated animals, contained little of this factor. The failure of the spreading factor to hydrolyze hyaluronidate by the turbidimetric method of assay and the difference in the inactivation temperatures of the mammary gland spreading factor as contrasted to the testicular spreading factor indicates rather definitely that the mammary gland spreading factor is not hyaluronidase.

The assay of mammary gland extracts of pregnant rats indicated that the highest level of spreading factor elaboration occurred at the time lobule-alveolar growth is known to be at a maximum.

It appears probable that a mucolytic enzyme or collagenase is elaborated by the cells of the growing mammary gland. Several mucolytic enzymes have been shown to have no hydrolytic action on hyaluronidate(2-4). Further, tumors which have long been reported to contain a spreading factor(8) after

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‡ Data on the spreading effect included in Table I.

8. Boyland, F., and McLean, D. A., *J. Path. and Bact.*, 1935, v41, 553.

much controversy have been reported not to contain hyaluronidase(9). Experiments are now in progress to determine the role of estrogen and progesterone upon the elaboration of the spreading factor by the mammary gland.

Summary. 1. Spreading type assays made on extracts of mammary glands taken from female albino rats pregnant from 3 to 18 days indicated the presence of a spreading factor. The concentration of the factor increased until about the twelfth day followed by a gradual decline. 2. Extracts of mammary glands from normal male and female rats, as well as castrate animals, assayed by the spreading method failed to exhibit any spreading effect at the dilution employed. 3. Turbidimetric assays of mammary gland extract showed that the spreading factor did not hydrolyze the substrate, hyaluronidate. 4. Tests showed that after heating at 60°C for 10 minutes, the spreading factor was still active but inactive after heating at 70°C for 10 minutes.

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Action of Adrenocorticotropic Hormone (ACTH) in Experimental Allergic Encephalomyelitis of the Guinea Pig. (18207)

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Recent observations in the experimental (1,2) and clinical(3) fields suggest that administration of ACTH inhibits allergic reactions. The type of encephalomyelitis elicited in the guinea pig by the injection of brain emulsion with adjuvants(4-7) provides a convenient laboratory example of an allergic condition since it can be produced easily with a single inoculation of antigen, follows a regular pattern, and shows distinctive clinical and pathological features. Therefore, it was considered of interest to investigate the effect of ACTH upon the occurrence and severity of this type of experimental allergic encephalomyelitis.

Material and methods. Eight groups of guinea pigs, each weighing 300-350 g were given a single injection of rabbit-brain emulsion with adjuvants in the manner previously described(5). Two groups served as controls and received no further treatment, whereas the other 6 groups received injections of ACTH. A purified hormone preparation was used which was assayed for ACTH potency by the ascorbic acid depletion method of Sayers(8). A typical assay of potency on this

TABLE I. Assay of Potency of ACTH Preparation

Dose inj. per 100 g rat body wt, γ	Decrease in ascorbic acid mg/100 g adrenal tissue
0.5	43
2.0	112
8.0	158

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1. Selye, H., *Canad. Med. Assn. J.*, 1949, v61, 553.
2. Berthrong, M., Rich, A. R., and Griffith, P. C., *Bull. Johns Hopkins Hosp.*, 1950, v86, 131.
3. *Proceedings of the First Clinical ACTH Conference*, Ed. by J. R. Mote, Blakston Co., New York, 1950.
4. Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, v57, 179.
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6. Cazzullo, C. L., and Ferraro, A., *Arch. Path.*, 1949, v48, 316.
7. Alvord, E. C., *J. Immunol.*, 1949, v61, 355.
8. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.

TABLE II. Effect of ACTH on Experimental Allergic Encephalomyelitis Induced in Guinea Pigs.

	Admin. of ACTH after inj. of brain emulsion			No. of guinea pigs that					
						Showed paralysis			
Group No.	No. of 5 mg doses	Total dose, mg	Days of inj.	No. of animals	Remained normal	Died	Recovered	Sacrificed	Pathologic lesions
468	13	65	1st-14th	10	10*	—	—	—	(See text)*
512	12	60	1st-13th	10	6	—	2	2	Minimal
511	4	20	5,8,11,15th	10	7	2	1	—	Mild
509	2	10	1st & 2nd	10	5	1	1	3	Mild to moderate
510	2	10	5th & 10th	9	6	—	—	3	Moderate to severe
508	2	10	10th & 11th	8	4	1	1	2	” ” ”
Controls									
469				9	1	1	2	5	Severe
513				11	2	3	4	2	”

* 5 animals were sacrificed for histologic examination; 2 showed no apparent lesions, in the other 3 very small subpia lesions and few single perivascular infiltrations were observed.

material is shown in Table I. It should be mentioned that the presence in this preparation of small amounts of pituitary hormones other than ACTH could not be excluded. The ACTH was administered by the subcutaneous route in a 5 mg (0.5 ml) dose, according to the schedule shown in Table II. The animals were kept on normal laboratory diet and examined daily for evidence of illness or paralysis. From each group 2 or more animals were examined histologically. With the exception of Group 468, those animals in each group which showed the most severe clinical manifestations of involvement of the central nervous system were selected for examination. The entire brain and spinal cord were fixed in formalin, embedded in paraffin, and cut serially at 25 μ. Each tenth section was stained with Einarson's gallocyanin method(9). A series of some 200 sections was thus examined in each case. This procedure enabled an adequate appraisal of the intensity and distribution of the lesions.

Results. It may be seen from the results summarized in Table II that high dosage of ACTH (total 65 mg), started on the day after the injection of brain emulsion and continued until the time when usually symptoms are apparent, protected all of the animals in Group 468, none of which developed clinical signs of the disease. Five animals from this group were sacrificed and studied histologically. Two showed no apparent lesions; in the other 3 very small subpia lesions and a very few single perivascular infiltrations were found which showed little tendency to spread into the surrounding tissue (Fig. 1a). It should be noted that in the specimens from the control animals almost every section showed multiple lesions (Fig. 1b). The contrast between treated and untreated animals was striking, from a pathological point of view. In the second group (512) of guinea pigs which had been given a similarly high dose of ACTH (total 60 mg), 4 developed paralysis; 2 of these recovered and 2 were sacrificed. The lesions seen were of about the small size as those observed in the preceding group except that they were more frequent in number. That a smaller dose (total 20 mg) still affords significant protection is shown by Group 511 in which 7 out of 10 animals showed no signs of the disease. The 2 animals of this series, sacrificed when paralyzed, showed small perivascular infiltration, more diffuse in distribution than in Group 512.

In an attempt to establish the minimal protective dose, and the time at which the hormone becomes effective, 3 experiments were carried out. Group 509 showed that some protection is obtained from small doses (total 10 mg), provided that the hormone is given early after the injection of antigen, as evidenced by the difference in the pathologic lesions seen in Groups 510 and 508 and those in Group 509. Although the number of guinea pigs affected in these 3 groups was not

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9. Einarson, L., *Am. J. Path.*, 1932, v2, 295.

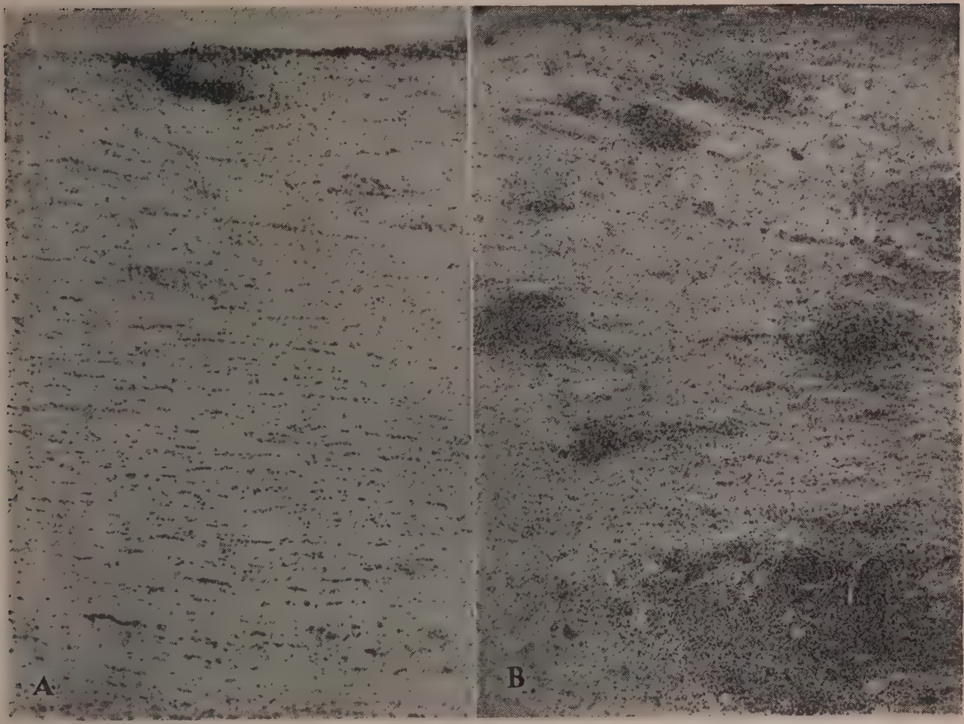

Fig. 1.

White matter of spinal cord—Einarson's stain low power. (a) Animal treated with ACTH showing only a small perivascular lesion. (b) Control animal showing extensive encephalitic lesions.

strikingly different, the pathologic lesions were much more severe and diffuse when the administration of ACTH was delayed until the 10th day after the injection of brain emulsion. Additional experiments are in progress, aiming at a more exact determination of effective dosage and time of administration of the hormone.

Comments. The available evidence on the biological effects of ACTH being still scanty and contradictory, it is premature to offer a satisfactory explanation of the mechanism of protection showed by ACTH in experimental encephalomyelitis of the guinea pig. It may be pointed out, however, that since allergic encaphalomyelitis is presumably associated with the formation of brain antibodies(10), the protective action may be due to the inhibitory effect of ACTH on antibody formation, as suggested by Fischel(11). In this connection it is of interest to note that other agents, such as nitrogen mustard(12) and salicylates(13), which are known to block antigen antibody reactions, offer some protection against the development of experimental encephalomyelitis of the guinea pig. Additional evidence in favor of the inhibiting action of the hormone on allergic phenomena is reported by Selye(1) who demonstrated that ACTH inhibits the anaphylactoid reaction brought about by the parenteral administration of egg white, and by Berthrong *et al.*

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10. Koprowski, H., and Jervis, G. A., Proc. Soc. Exp. Biol. and Med., 1948, v69, 472.

11. Fischel, E. E., *Bull. N. Y. Acad. Med.,* 1950, v26, 255.

12. Koprowski, H., Jervis, G. A., and Black, J., Unpublished experiments.

13. Good. R. A., Campbell, B., and Good, T. A., Proc. Soc. Exp. Biol. and Med., 1949, v72, 341.

(2) in experiments on vascular hypersensitivity.

On the other hand, the mechanism of the action of ACTH in allergic encephalitis might be purely at a vascular level. It has been shown by Soffer *et al.*(14) that ACTH suppresses the Schwartzmann phenomenon when administered prior to the provocative injection. This phenomenon is admittedly not anaphylactoid in nature, and its cause is to be found in a profound alteration of vascular reactivity(14). Pathologic studies of allergic encephalomyelitis(5) show that the lesion begins in and around the blood vessels from which it spreads into the nervous parenchyma. Therefore, it is possible that by modifying the vascular reactivity in a manner similar to that observed by Soffer(14), ACTH protects the nervous tissue against the development of encephalitis lesions. This hypothesis is, at present, the object of further investigation in guinea pigs and in monkeys.

Although the relation of experimental encephalomyelitis in animals to human disease is still controversial, it would be of interest to investigate the action of ACTH, or of cortisone, in the prophylaxis of post-vaccination encephalitis and in the prevention of relapses of certain acute forms of multiple sclerosis, two types of human disease in which pathogenic factors similar to those operating in the experimental encephalomyelitis of the guinea pig are presumably playing a role.

Summary. Timely administration of adequate doses of ACTH shows an inhibiting action upon the occurrence of clinical manifestations and the development of pathological lesions in experimental allergic encephalomyelitis of the guinea pig.

The authors wish to express their appreciation to Miss Maja Platzer for her assistance in the preparation of the manuscript.

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14. Soffer, L. J., Schwartzman, G., Schneierson, S. S., and Gabrilove, J. L., *Science*, 1950, v111, 303.

The Determination of Radioactive Iodine in Biologic Material.* (18208)

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Most of the methods for the determination of radioiodine in the presence of organic material are insensitive and time-consuming. The purpose of this paper is to describe a method which obviates to an appreciable extent these disadvantages.

Reagents and apparatus. Sulfuric acid, 18 N; potassium permanganate; oxalic acid, saturated aqueous solution; lithium arsenite, a solution containing 0.74 g of arsenious oxide (As_2O_3) and 1.20 g of lithium hydroxide (LiOH) per 100 cc of solution; modified Chaney distillation unit(1,2); Geiger-Müller beta ray counter, with appropriate scaler.

Experimental. The material (blood, urine or tissue) to be analyzed is placed in a 500 cc pyrex digestion flask to which is added 1 g of potassium permanganate and 18 cc of 18 N sulfuric acid for each 50 mg of organic material. (Caution: The addition of undiluted concentrated sulfuric acid to permanganate is dangerous.) As carrier, 1 cc of a solution containing 1 mg of sodium iodide per cubic centimeter is added to the flask. The flask is heated by either a gas flame or an electric heater until dense white fumes form (186°-190°C). The flask is then cooled, 25 cc of distilled water is added and

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* Part of a thesis submitted by Dr. Rall, to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine.

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2. Talbot, N. B., Butler, A. M., Saltzman, A. H., and Rodriguez, P. M., *J. Biol. Chem.*, 1944, v153, 479.

TABLE I. Recovery Experiments with I^{131} by Distillation Procedure.

Material			Controls			Experimental			
Solvent	I^{131}, μc	I^{127} μg	Samples	Mean counts per sec.	Stand. error	Samples	Mean counts per sec.	Stand. error	Diff.
Radioiodide									
Water	.02	.1	5	36.91	±.35	10	38.01	±1.40	+1.10 ± 1.44
”	.02	.1	4	30.10	±.32	10	28.27	± .36	—1.83 ± .48*
”	.02	.1	4	46.90	±.33	10	43.00	± .49	—3.90 ± .58*
Urine	.02	.8	4	54.53	±.46	10	52.24	± .59	—2.29 ± .75*
Serum	.015	.04	4	38.82	±.68	10	36.55	+ .30	—2.27 ± .74*
Radiothyroxine									
Water	.009	.25	4	30.01	±.28	10	30.38	± .20	+0.37 ± .34
Urine	.01	1.4	4	35.03	±.35	10	36.00	± .42	+0.97 ± .55
Serum	.008	.4	4	23.48	±.18	10	23.63	± .12	+0.15 ± .22
Radiodiiodotyrosine									
Water	.01	.1	4	30.35	±.22	10	29.83	± .19	—0.22 ± .29
Urine	.018	1.55	4	51.48	±.74	10	51.19	± .34	—0.29 ± .81
Serum	.012	.19	4	36.76	±.39	10	35.51	± .46	—2.25 ± .60*

* A significant loss.

distillation into a Chaney still(1) started. As moisture appears in the trap of the distilling unit, 1 cc of lithium arsenite solution is introduced into the trap, after which the condenser is attached. When the water starts to condense and return to the digestion flask, 10 cc of oxalic acid solution is added dropwise. Distillation is continued for 5 minutes after decolorization of the permanganate occurs. At the end of this period of distillation, the fluid in the trap is drained into a small beaker and the trap plus receiver rinsed 3 times with 2 cc of distilled water. The beaker is placed on a steam bath until the water is completely evaporated. The residue is then taken up in 2 cc of water, and ½ or ¾ of the solution transferred to a nickel-plated cup planchette. The planchettes are dried under an infra-red heater and the radioactivity determined by a thin mica window beta ray counter and scaler. The amount of radioactivity represents ½ or ¾ of the amount in the original sample and the appropriate correction is made. No correction is needed for self-absorption since there are only 10 to 15 mg of solids present. Computation in terms of microcuries of I^{131} is made in the usual way by simultaneous comparison with a sample of a standard.

Results. The procedure was evaluated by performing recovery experiments using radioiodide, radiothyroxine and radiodiiodotyrosine added to water, urine, serum and various animal tissues. The total amount of I^{127} in these solutions or mixtures was so adjusted as to be approximately the same as that ordinarily present in these tissues or fluids. The purpose of this precaution was to evaluate adsorption losses, since such losses on glassware such as pipets may be appreciable when small amounts of iodine are handled. The amount of radioactivity in all experiments varied from .01 to .02 microcurie since this amount of radioactivity gave a counting rate of from 40 to 100 times background. The solutions were made up so that in the case of water and urine 5 cc aliquots were transferred to the digestion flasks and in the case of the serum 1 to 2 cc aliquots were used. In each recovery experiment 10 aliquots were run. As controls, aliquots containing the same amount of radioactivity were added to a mixture of 1 cc of lithium arsenite solution plus 10 cc of water and the solution evaporated over the steam bath in precisely the same procedure as used for the experimental runs. The results of these recovery experiments are shown in Table I. The overall recovery varied between 94 and 103%. The uniformity of the individual values, indicated by the small standard error of the mean, was such that a loss of 4 to 5% indicated a significant loss. The values that represent significant losses are starred in Table I.

The recovery of I^{131} in the presence of

TABLE II. Recovery of I^{131} in the Presence of Tissue.

Tissue	Wt of sample, g	Recovery of I^{131}, %
Skin	1	101.7
Adipose tissue	1	98.1
Liver	1	99.3
Spleen	1	90.6
Kidney	1	96.5
Adrenal	.073	96.5
Ovary	.053	116.2
Pituitary	.014	99.7
Brain	1	100.6
Smooth muscle (ileum)	1	96.4
Striated muscle	1	91.5
Blood	1	92.3
Thymus	.55	98.5
Thyroid	.017	93.7
Feces	1	93.1

various tissues was determined by the addition of 1 cc of a solution containing .008 microcurie of I^{131} and 0.1 μg of I^{127} per cubic centimeter to 15 tissues of the rat. Various tissues were chosen because the presence of volatile substances in certain tissues might easily affect the recovery of I^{131}. The results of this experiment are shown in Table II, which indicates that satisfactory recovery was achieved in each instance in spite of the fact that only one sample was determined.

Comment. The procedure just described is an adaptation and a modification of the Chaney method for the chemical determination of iodine in biologic material with a few changes necessary for isotope measurement. The method has proved to be useful because of its convenience and sensitivity. Twenty samples can be digested and distilled conveniently and the radioactivity determined the following day. Because of the small amount of solids in the distillate, very satisfactory planchettes[†] always result, and corrections for self-absorption are unnecessary. The over-all sensitivity of this method was computed to be 30% by comparison with a uranium beta ray standard, that is, 30% of the beta radiation of the sample is recorded by the apparatus. With this sensitivity, as little as 3 x 10^{-5} microcurie of radioiodine per cubic centimeter of serum may be determined accurately at a counting rate 10 times the background. It is therefore possible to determine the concentration of I^{131} in serum of patients during a half-life of I^{131} following administration of doses of about 20 microcuries of radioiodine.

Summary. A convenient and accurate method for the measurement of small amounts of I^{131} in biologic material is described.

[†] For planchettes nickle-plated cups 2.5 cm in diameter and 0.25 cm deep are used. These are placed in a brass holder at a distance of 8 mm from the Geiger-Müller tube. The Geiger-Müller tube is shielded throughout by an inch of lead. With this shielding the cosmic and incidental radiation background varies from 0.2 to 0.3 count per second. The shielding for the Victoreen tube was designed by Dr. M. M. D. Williams, Division of Biophysics and Biophysical Research, and Mr. Dana Rogers, Division of Engineering, Mayo Clinic, to both of whom the authors are greatly indebted.

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Replacement of Pantothenic Acid by the *Lactobacillus bulgaricus* Factor in Rat Nutrition.* (18209)

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(Introduced by G. Howard Satterfield)

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The existence of a factor essential for the growth of *Lactobacillus bulgaricus* (Sarles) and *Lactobacillus helveticus* 80 has been re-

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TABLE I. Mean Weight Gains in Rats on Diets Supplemented with Calcium Pantothenate and LBF Concentrate.

Diet No.	Daily supplement	No. of survivors	Gain in wt, g 5 days	 7 days	 10 days	 12 days
1	None	0	—	—	—	—
2	Ca pantothenate 12.5 μg	4	8 ± .9*	10.5 ± .7	16 ± 1.2	21.3 ± 3.3
3	" " 25 "	6	10 ± .5	12.3 ± .6	20.7 ± 1.4	27 ± .9
4	" " 50 "	8	10.3 ± 1.0	13.9 ± .6	19.4 ± .8	29.5 ± .8
5	" " 100 "	8	11 ± .3	15.4 ± 1.0	18.5 ± 1.4	30.8 ± 1.3
6	" " 200 "	8	11.5 ± .7	16.2 ± 1.0	20.5 ± .9	30.4 ± .9
7	LBF concentrate 3200 units† (equivalent to 96 μg pantothenate)	8	10.2 ± .8	17.1 ± 1.3	18 ± 1.3	29.3 ± .8
8	LBF conc. 6400 units (equivalent to 192 μg pantothenate)	8	9.5 ± .6	16.1 ± .4	16.8 ± 1.2	29.1 ± 1.0

* Mean ± stand. dev. of the mean.

† The unit of LBF is defined as that amount contained in 1 mg of standard Basamin-Busch yeast extract(1).

ported recently(1). This factor (LBF) has subsequently been shown to be essential for several other strains of the more fastidious lactic acid bacteria(2,3) and to be interchangeable with pantothenate for *Lactobacillus casei* and *Lactobacillus arabinosus*(4).

Partial identification indicates that LBF contains pantothenate in a bound form that is 50 to 100 times more active than free pantothenate for organisms which require LBF (4). Rasmussen *et al.*(5) have suggested that LBF may be required for the maximum growth of chicks on a vegetable-protein basal diet supplemented with fish solubles and vitamin B_{12}. This study was undertaken to determine the ability of the rat to utilize LBF on pantothenate-deficient diets.

Experimental. Rats of the Wistar strain, 22 to 24 days old, were placed on a basal diet consisting of 65% glucose, 18% casein, 10% fat, 4% minerals (USP No. 2), 2% cellulose, and 1% cod-liver oil. This was supplemented with 20 μg each of thiamine, riboflavin, pyridoxine, and 5 mg of choline chloride per rat per day. This is essentially the diet of Unna(6) and has been found adequate in our laboratory when supplemented with pantothenic acid. Rats on this regimen lost weight at an average of 0.5 g per day during the depletion period of 21 to 23 days. Prior to feeding the test diets, rats were selected as to weight and sex and placed in individual cages on wire floors. Graded doses of calcium pantothenate and two levels of LBF concentrate were fed in addition to the basal diet and vitamin supplement. The vitamin supplements and the test levels of calcium pantothenate or LBF concentrate were mixed with the basal diet in such proportion that 2 g contained the daily dosage. The supplemented test diets and unsupplemented basal diet were weighed out to the rats daily in flanged cups to prevent spillage. Uneaten food was weighed and the amount of unsupplemented basal diet fed was adjusted to insure daily ingestion of the supplemented test diet. Eight rats, 4 males and 4 females, were fed each test diet. Four rats, 2 males and 2 females, served as controls. The rats were weighed at intervals of 2 to 3 days and the gains are recorded in Table I. The LBF concentrate was prepared from *Bacillus megatherium* culture filtrate by adsorption on charcoal and elution with *n*-butanol.

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It contained 38,500 LBF units per ml and had a potency of 840 units per mg of dry solids. Diet 7 contained 3,200 LBF units in a daily supplement and was equivalent to 96 μg of bound calcium pantothenate as determined by alkaline hydrolysis and microbiological assay(7) of the β-alanine liberated. Paper chromatography of materials containing LBF has shown that 5 or more substances possess LBF activity(5,8). The concentrate used in this study consists mainly of a mixture of the 2 LBF's (Rf 0.83 and 0.74) obtained in concentrates from *B. megatherium* culture filtrates(8). Less than 2 μg of free pantothenate were contained in a daily supplement. Diet 8 contained twice these amounts of free pantothenate and pantothenate bound in the LBF molecule.

Results and discussion. There was no significant difference between the growth responses produced by the 4 highest levels of calcium pantothenate and either level of LBF concentrate at the end of 12 days. This merging of response is to be expected since a daily dose of 25 to 50 μg of Ca pantothenate appears to be adequate for optimum growth after the rat has recovered from severe depletion(6).

Diet 8 had a pronounced odor and was not readily consumed by the rats. Subnormal gains on this diet were probably the result of inadequate food intake due to unpalatability, although the possibility of toxicity due to impurities in the concentrates must also be considered.

Controls and some of the rats on the low pantothenate diets died in 3 to 4 days after the start of the experiment, as shown in Table I. All rats receiving over 50 μg of calcium pantothenate or the LBF supplements survived. This leaves little doubt as to the replacement of pantothenate by LBF for the rat. Further proof of this interrelationship is evidenced by the fact that rats which were prostrate after a depletion of 24 to 25 days were on their feet and had apparently recovered in a few hours after intraperitoneal injection with LBF concentrates or calcium pantothenate. Death occurred in 1 to 2 hours after prostration in uninjected rats.

Summary. Evidence is presented to show that concentrates of the *Lactobacillus bulgaricus* factor will replace pantothenic acid in the nutrition of the rat. The quantitative effect of LBF in promoting growth may be in excess of that expected from the pantothenate contained in the LBF molecule.

We are indebted to Dr. J. C. Lewis, Western Regional Research Laboratory, Albany, Calif., for the *B. megatherium* growth liquors and to Mr. C. L. Long of this department for the paper chromatographic analysis of the LBF concentrate.

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Occurrence of Tyrosinase in Horse and Fish Melanomas. (18210)

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In addition to the presence of melanomas in man, these tumors have been reported in the horse, mule, cow, swine, goat, dog, cat, chicken, rabbit, mouse, snake, and fish(1-6). Despite this widespread occurrence in animals, particularly in horses(1,2), there are relatively few reports on the biochemical properties of these tumors. It has been found that extracts from horse melanomas(7,8) catalyze the conversion of tyrosine to melanin, and that extracts from the Harding-Passey mouse melanoma(9,10) catalyze the formation of melanin from both tyrosine and 3,4-dihydroxyphenyl-L-alanine (dopa). Alsberg(11) prepared an extract from a human melanoma which catalyzed the formation of black pigment from catechol and possibly from tyrosine. Neuberg(12) showed that dilute extracts from a human melanoma accelerated pigment formation from tyramine and epinephrine but not from tyrosine. Greenstein and others(13) found that extracts from a human melanoma showed both tyrosinase and dopa-oxidase activity. We have shown(14) that extracts of the Harding-Passey mouse melanoma are equally active in catalyzing the enzymatic oxidation of both tyrosine and dopa under the proper conditions, and we suggested that the general term "tyrosinase" be used for the enzyme or enzyme complex concerned instead of the more restrictive terms "tyrosinase" and "dopa-oxidase." We further demonstrated(15) that tyrosinase from mouse melanoma, like tyrosinase from plants(16) and insects(17), requires copper for its activity and is inhibited by diethyldithiocarbamate and other compounds having an affinity for copper.

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This report describes the enzymatic oxidation of tyrosine and dopa by extracts of horse and fish melanomas, and the inhibition of this oxidation by diethyldithiocarbamate.

Experimental methods. Preparation of the tumor extracts. (A) Horse melanoma. The specimen,§ the largest of a group of firm nodular and ovoid tumors located in the skin of the ventral surface of the tail, anal and perineal regions, was removed from the right rectal fossa. The tumor weighed approximately 500 g, and was coal-black in color. After removal, the tumor was frozen with solid carbon dioxide until used 3 hours later. To prepare the extract, approximately 2 g of the melanomatous tissue were ground with sand and cold 0.1 M potassium phosphate buffer, pH 6.8, for 10 minutes. The resulting black, syrupy material was allowed to stand for 12 hours at 5°C. The watery supernatant fraction was then decanted from the large clumps at the bottom of the vessel and used for the enzymatic studies.

(B) Fish melanoma. The melanoma-bearing fish¶ used in the experiments were first generation hybrids between a male spotted-belly platyfish *(Platypoecilus maculatus)* and an albino swordtail *(Xiphophorus helleri)*. Gordon(6) has demonstrated that there is a very high incidence of spontaneous melanomas in such offspring.

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§ The gray mare from which this tumor was removed was supplied by Sharp & Dohme, Inc., Philadelphia, Pa., through the courtesy of Drs. R. H. Barnes and B. J. McGroarty. The animal was estimated to be 17 years old.

¶ The fish were obtained from the aquarium of the New York Zoological Society through the courtesy of Dr. Myron Gordon.

The fish were killed by chilling in ice and were kept in ice until used, 2 days later. The deeply pigmented tumors, each weighing approximately 50 mg, were removed by blunt dissection and were ground in sand and 5 ml of cold 0.1 M potassium phosphate buffer at pH 6.8 for 5 minutes at room temperature. The material was then centrifuged at 2,500 revolutions per minute for 20 minutes at 5°C. The clear, red-brown supernatant fraction, containing 0.5 mg of nitrogen per ml, was stored at 5°C until used one to 2 hours later.

Enzymatic oxidation of L-tyrosine and L-dopa was measured manometrically as oxygen uptake in the Warburg apparatus, at 38°C and in 0.1 M potassium phosphate buffer at pH 6.8, using 0.2 ml of horse melanoma extract or 0.5 ml of fish melanoma extract per vessel, in a total fluid volume of 3 ml. The substrates (tyrosine, dopa) were added in 0.5 ml of buffer from the vessel side arms after 10 minutes equilibration, and manometric readings made at 10 minute intervals thereafter. Sodium diethyldithiocarbamate was dissolved in buffer to give 0.3-0.5 mg per vessel and added directly to the enzyme solution before equilibration. In the experiments involving heavy metals in the presence of diethyldithiocarbamate, a 25% excess of the various metal salts, calculated on a mole for mole basis, was dissolved in 0.1 ml of water and added to the reaction mixture from a second side arm after the reaction had been allowed to run for 30 minutes. The metal salts used were cupric sulfate, ferric nitrate, magnesium chloride, zinc sulfate, nickelous sulfate, cobaltous sulfate and manganous chloride.

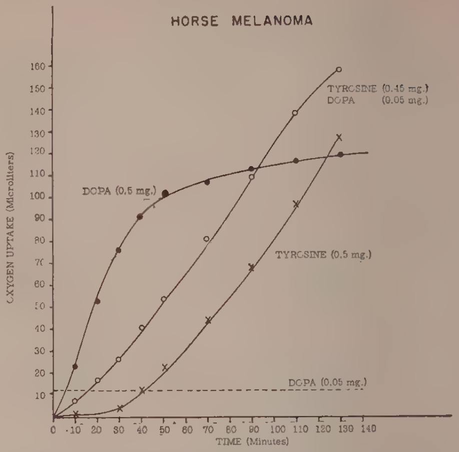

FIG. 1.

Enzymatic oxidation of tyrosine and dopa by an extract from the horse melanoma at pH 6.8 and 38°C.

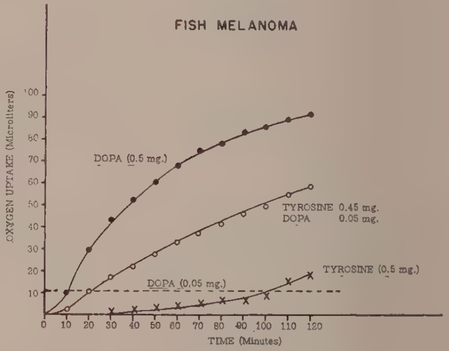

FIG. 2.

Enzymatic oxidation of tyrosine and dopa by an extract of fish melanoma at pH 6.8 and 38°C.

Results. Fig. 1 and 2 illustrate the varying rates of oxygen uptake when tyrosine, dopa, and tyrosine plus a trace amount of dopa, were used as substrates with extracts of the horse and fish melanomas. It is evident that the 2 extracts have qualitatively the same enzymatic activity. With dopa as the substrate, oxygen uptake began almost immediately. With tyrosine, however, oxygen uptake did not commence until after an interval of approximately 30 minutes (horse melanoma extract) and 90 minutes (fish melanoma extract). Substitution of 0.05 mg of dopa for an equal quantity of tyrosine markedly shortened this interval and there was a rapid oxidation of tyrosine. The added dopa by itself can account for an oxygen uptake of not more than 11 microliters as indicated by the dotted horizontal lines in Fig. 1 and 2.

A preparation of the horse melanoma extract, made by the method described above, except that the extract was centrifuged at 3,000 r.p.m. for 30 minutes at 5°C, yielded a brown supernatant fraction which had no enzymatic activity. This suggests that en-

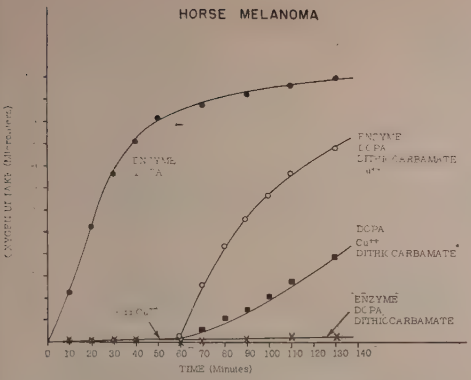

Fig. 3.

The effect of sodium diethyldithiocarbamate (0.5 mg) on the enzymatic oxidation of dopa (0.5 mg) by an extract from the horse melanoma and the reversal of inhibition by Cu^{++} (0.9 mg $CuSO_4 \cdot 5\ H_2O$); pH 6.8 and 38°C.

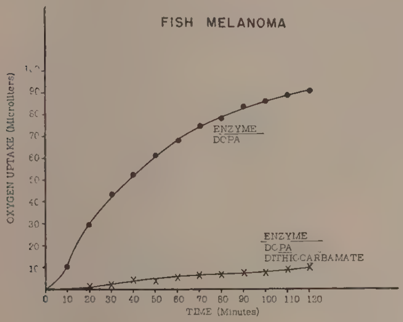

Fig. 4.

The effect of sodium diethyldithiocarbamate (0.3 mg) on the enzymatic oxidation of dopa (0.5 mg) by a fish melanoma extract at pH 6.8 and 38°C.

zymatic activity is associated with insoluble particulate matter of the cell, as has been shown for mouse melanoma(14). However, the mouse melanoma enzyme is not separated from the supernatant fluid under these particular conditions, indicating that the particulate material in the horse extracts is larger than that in the mouse extracts. Tissue slices of the horse tumor showed the same enzymatic activity as the uncentrifuged extract.

Sodium diethyldithiocarbamate almost completely inhibited the uptake of oxygen by mixtures of horse tumor extract and substrate. This was reversed by the addition of cupric ions (Fig. 3). The catalytic effect of cupric ions on the auto-oxidation of dopa is also illustrated. Reversal of diethyldithiocarbamate inhibition did not occur on the addition of manganous, ferric, magnesium, zinc, nickelous and cobaltous ions.

The marked inhibition of oxygen uptake produced by sodium diethyldithiocarbamate in the mixtures of fish tumor extract and substrate is shown in Fig. 4. An attempt to reverse this inhibition by the addition of an excess of cupric ions did not yield conclusive results. Unfortunately there was insufficient material for the detailed controls necessary to clarify this point. Attempts to demonstrate tyrosinase activity in extracts from normal pigmented and non-pigmented skin of fish of the same species yielded negative results.

The foregoing experiments demonstrate the presence of tyrosinase in extracts of horse and fish melanomas. The pattern of enzymatic activity is similar to that reported(14) for the tyrosinase in extracts of mouse melanoma, particularly with respect to the catalytic effect of dopa on the oxidation of tyrosine. Inhibition of tyrosinase activity by diethyldithiocarbamate, and its specific reversal by added cupric ions, strengthens the view that horse melanoma tyrosinase (and probably the fish melanoma enzyme also) requires copper for enzymatic activity, as does the mouse melanoma enzyme(15).

Summary. 1. A crude extract with marked tyrosinase activity has been prepared from melanomas of the horse and certain species of hybrid fish. These extracts catalyzed the oxidation of both tyrosine and dihydroxyphenyl-L-alanine (dopa) to melanin. In addition, dopa has a catalytic role in the enzymatic oxidation of tyrosine by these extracts.

2. Diethyldithiocarbamate, a substance with a strong affinity for copper, produces a marked inhibition of the enzymatic reaction. This inhibition is reversed by cupric ions in the case of the horse melanoma extracts. This reversal is not obtained with other metals.

3. The enzymatic activity of horse melanoma extracts appears to be associated with cellular particulate matter, which is larger than that found in mouse melanoma extracts.

4. The biochemical properties of the tyrosinase preparations from the horse and fish melanomas are similar to the properties of the preparation obtained from mouse melanoma.

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Effect of Adrenocorticotrophic Hormone, Cortisone and Desoxycorticosterone on Brain Excitability.* (18211)

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It has been demonstrated in this laboratory that desoxycorticosterone acetate (DCA) decreases brain excitability as measured by the electroshock seizure threshold (EST)(1,2), and that brain excitability of DCA-treated rats can be restored to normal by the administration of adrenocorticotrophic hormone (ACTH) or adrenocortical extract (ACE)(2). The following experiments are an extension and elaboration of these earlier studies.

Methods. Adult male rats from the Sprague-Dawley farm were employed. The technic for determining EST has been presented in detail(3). EST measurements were made at 2- to 3-day intervals for a period of about two weeks before the beginning of hormone administration. Thresholds tend to be more variable and slightly higher at the beginning than at the end of such a period of stabilization. Hence, the alterations in EST observed throughout the course of the experiments have been expressed as a percentage of the control value obtained at the *end* of the stabilization period. When thresholds had stabilized, some of the rats were unilaterally nephrectomized, implanted subcutaneously with DCA† (twelve 15-mg pellets per rat) and given a 0.9% solution of sodium chloride to drink. EST measurements were made every 2 to 3 days throughout the experiments. The dose of ACTH‡ is expressed in all instances as the mg equivalent of the ACTH standard, La-1-A. Preparation 74 Ax, employed in Experiment A, is 50% as potent as La-1-A; it has posterior pituitary pressor activity of less than 0.06 unit per mg equivalent of La-1-A. Preparation 83 ASI, employed in Experiment B, is 160% as potent as La-1-A; it has posterior pituitary pressor activity of less than 0.05 unit per mg equivalent of La-1-A. ACTH was dissolved in a 0.9% solution of sodium chloride at pH 1; each dose was injected subcutaneously in a volume of 0.2 ml. Cortisone acetate was suspended in 10% ethanol; each dose was injected subcutaneously in a volume of 0.2 ml. Posterior pituitary substance (Pitressin, Parke, Davis and Co., 20 pressor units per ml) was diluted with 0.9% sodium chloride solution to a concentration of 0.1 unit per 0.2 ml; this volume was injected subcutaneously. When combinations of hormones were employed, the preparations were injected throughout the entire course of the experiment, starting at the time of DCA implantation. This is in contrast to the previous study(2) in which ACTH and ACE were not injected until the rats had been under the influence of DCA for some time.

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* This investigation was supported by research grants from the Division of Research Grants and Fellowships of the National Institutes of Health, the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and the Upjohn Co.

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‡ ACTH was obtained through the kindness of Dr. E. E. Hays of the Armour Co.

TABLE I. Treatment of Experimental Groups.

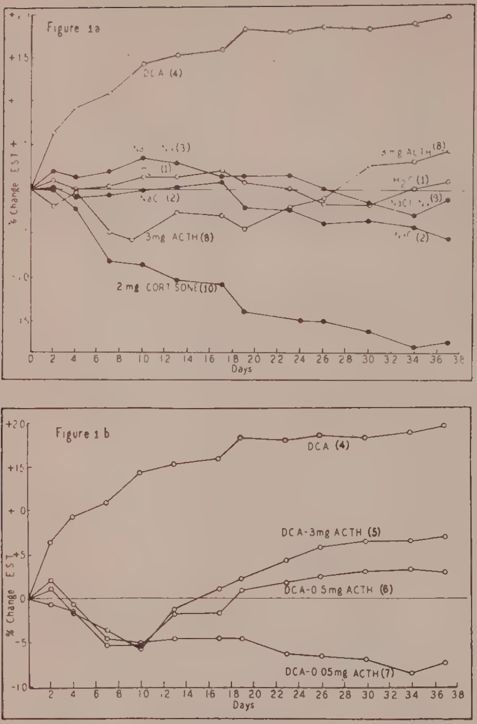
Exp.	Group	No. rats	Unilateral nephrectomy	Drinking fluid	DCA 15 mg pellets	ACTH* total dose, mg	Cortisone,* total dose, mg	Initial body wt, g and ± S.E.	Final body wt, g and ± S.E.
A	1	4	No	H_2O	0	0	0	350 ± 19	380 ± 25
	2	5	”	NaCl	0	0	0	350 ± 17	375 ± 16
	3	6	Yes	”	0	0	0	342 ± 18	368 ± 17
	4	5	”	”	12	0	0	337 ± 9	305 ± 18
	5	6	”	”	12	3(3)	0	345 ± 6	322 ± 9
	6	6	”	”	12	.5(1)	0	306 ± 5	278 ± 14
	7	5	”	”	12	.05(1)	0	344 ± 15	348 ± 12
	8	4	”	”	0	3(3)	0	325 ± 20	313 ± 20
	9	5	”	”	12	0	2(2)	336 ± 6	260 ± 8
	10	6	”	”	0	0	2(2)	333 ± 11	270 ± 12
B	11	7	”	”	0	0	0	263 ± 5	353 ± 6
	12	7	”	”	12	0	0	273 ± 4	319 ± 8
	13	7	”	”	12	.001(1)	0	266 ± 6	298 ± 11
	14	7	”	”	12	.01(1)	0	271 ± 6	312 ± 8
	15	6	”	”	12	10(5)	0	277 ± 9	256 ± 11
	16†	7	”	”	12	.5(1)	0	274 ± 7	323 ± 9
	17	7	”	”	12	0	.1(1)	263 ± 12	307 ± 13

* Numbers in parentheses indicate number of divided doses per day.
† 0.1 unit posterior pituitary 5 times daily.

Results. Two experiments, A and B, were performed. Information regarding the nature of the drinking fluid, hormone treatment and body weight changes of each group of rats is presented in Table I.

Fig. 1 illustrates the data obtained. Fig. 1a and 1c indicate that control rats, *i.e.*, Groups 1, 2 and 3 of Experiment A and Group 11 of Experiment B, showed little or no change in EST throughout the course of the experiment. A slight elevation of EST occurred in Group 3 of Experiment A from the 8th to the 12th day and a slight decline of EST occurred in Group 2 of Experiment A beginning on the 20th day. It may be concluded that excess dietary sodium and unilateral nephrectomy do not appreciably influence brain excitability in rats as measured by EST. In both Experiments A and B, DCA induced an elevation in EST similar to that previously reported from this laboratory(1,2). In the earlier studies rats with intact kidneys were implanted with six 15-mg pellets of DCA and were given water to drink. It thus appears that an increase in the dose of DCA from 6 to twelve 15-mg pellets, combined with unilateral nephrectomy and 0.9% sodium chloride solution to drink had no additive effect on brain excitability. It is of interest in this connection that the concentration of sodium in the plasmas of rats in Experiment A (153.2 ± 3.9 mEq per kg of plasma) was not significantly greater than the concentration of this cation (151.2 ± 1.2) in the plasma of rats implanted with six 15-mg pellets and given water to drink. The rats given ACTH alone in a dose of 1 mg thrice daily showed a slight initial decrease in EST, followed by a return to normal and a subsequent slight increase (Fig. 1a).

All doses of ACTH effectively antagonized the action of DCA on EST (Fig. 1b and 1c). The EST actually decreased below control levels during the first 10 to 12 days in all groups of DCA-rats treated with ACTH. The EST remained at a level lower than normal during the rest of the experimental period in rats given 0.01 mg, 0.05 mg or 10 mg of ACTH daily (Fig. 1b and 1c). At the 0.001 mg dose level (Fig. 1c) the EST returned to normal during the subsequent course of the experiment. A progressive rise in EST, beginning on the 10th day, occurred in DCA-rats treated with 0.5 mg or 3 mg ACTH (Fig. 1b). With the 3 mg dose the EST was significantly increased above normal during the



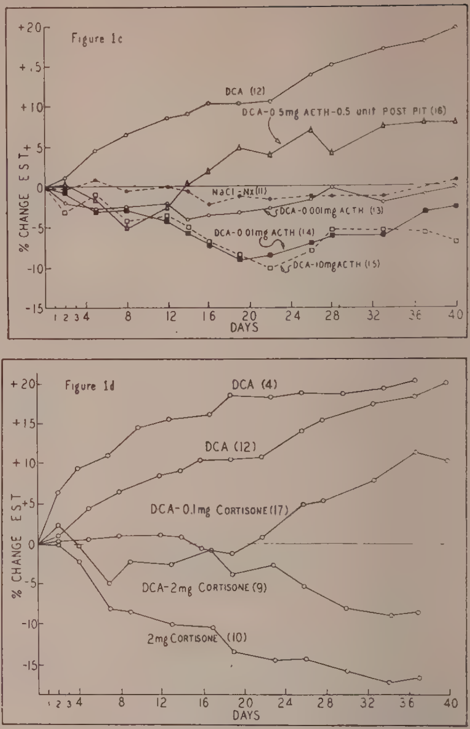


FIG. 1a and b. Average changes from control level (0) in electroshock seizure threshold (EST) with time in rats given various treatments. See text for explanation. Nx = unilateral nephrectomy. Numbers in parentheses are the group numbers (Table I).

FIG. 1c and d. Same legend as for previous figures.

last 14 days of the experiment, but the value did not approach that of animals given DCA alone. In DCA-rats treated with 0.5 mg ACTH plus 0.5 unit of posterior pituitary (Fig. 1c), the pattern of change in EST was similar to that of the DCA-animals treated with 3 mg ACTH (Fig. 1b).

Cortisone alone in a dose of 1 mg twice daily induced a marked reduction in the EST (Fig. 1d). Furthermore, this steroid effectively antagonized the EST-elevating properties of DCA (Fig. 1d). In a dose of 2 mg the action of the cortisone predominated and the ultimate EST of animals treated with the combination was significantly less than normal. Cortisone in a dose of 0.1 mg once daily maintained the EST of DCA-treated animals at a normal level until the 22nd day when there began a progressive increase in EST which, at the end of the experiment, was 10% above normal.

Discussion. A number of factors have been shown to affect the excitability of the brain as measured by the EST. The concentration of extracellular sodium has an important influence, the EST varying directly with the concentration of plasma sodium. Adrenalectomized rats have a hyponatremia in association with a decreased EST(3). DCA or sodium chloride restores to normal both the concentration of plasma sodium and the EST of adrenalectomized rats(3); DCA induces a hypernatremia and an elevation in EST in intact(1,2) and adrenalectomized(2) rats. Furthermore, both the hypernatremia and the decreased brain excitability induced by DCA are corrected by ACTH(2).

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Nutritional factors also have a pronounced effect on the excitability of the brain. It has been demonstrated in this laboratory(4) that restricted caloric intake, accompanied by failure to grow or loss in body weight, results in a marked reduction in EST. Although loss of weight in starved animals is accompanied by an increase in concentration of plasma sodium, there is a marked decrease in the EST(4,5). It is thus apparent that the influence of extracellular sodium on brain excitability can be antagonized, or even overshadowed by other factors.

The animals in Exp. A and B were given 0.9% sodium chloride solution to drink, whereas in a previous study(2) the animals were given water to drink.§ As pointed out above, ACTH prevented the hypernatremia and the elevation in EST in DCA-rats given water to drink. As yet incomplete electrolyte data indicate that there is no significant difference in extracellular sodium concentration among the various experimental groups, despite marked differences in EST; the available electrolyte data suggest that ACTH and cortisone can antagonize the action of DCA on brain excitability without producing an accompanying decrease in the concentration of plasma sodium.

In the previous experiments reported from this laboratory(2), ACTH and ACE were administered after EST had been elevated by DCA. It was demonstrated that ACTH and ACE could reverse the change in brain excitability induced by DCA. In the present experiments, it is demonstrated that the EST-elevating action of DCA can be prevented by ACTH or cortisone administered during the entire course of the experiment. ACTH, therefore, can both prevent and reverse the effect of DCA on the excitability of the central nervous system. It is likely that cortisone and ACE can also both prevent and reverse the action of DCA, although additional studies are required definitely to establish this point.

All doses of ACTH employed, 0.001 to 10 mg per day, completely antagonized the effect of DCA during the early phase of the experiments. Subsequently, the intermediate doses were not as effective as either the small or the very large doses of ACTH in lowering the EST of DCA-treated animals. It is remarkable that a dose of ACTH as small as 1.0 μg daily is effective. A dose of 10 μg of ACTH appears to have a slightly greater effect than 1.0 μg. The possibility that the ACTH-DCA antagonism may provide the basis of a new method of assay for ACTH is undergoing experimental test in this laboratory.

The secondary rise in the EST of animals treated with 0.5 and 3 mg doses of ACTH cannot be adequately explained at the present time. Several possible explanations have been considered, three of which are the following: (1) antihormone formation; (2) contamination of ACTH with a substance which influences brain excitability, *e.g.*, posterior pituitary; (3) changes in the qualitative nature of the secretion of the adrenal cortex. The data suggest that the first 2 possibilities are unlikely. The third is difficult to rule out in the present state of our knowledge regarding the nature of the secretion of the adrenal cortex.

That the 10 mg dose of ACTH acted similarly to the 0.001, 0.01 and 0.05 mg doses emphasizes the fact that numerous variables are operating to alter the EST in these experiments. It was pointed out above that restriction of food intake results in a marked reduction in the EST. The 10 mg dose of ACTH exerted a considerable catabolic effect as shown by the weight loss (–21 g, Group 15, Table I) in animals that normally grew at a rapid rate (+90 g, Group 11). The 0.5 and 3 mg doses of ACTH induced relatively less change in body weight (–28 g, Group 6 and –23 g, Group 5) in comparison to controls (+26 g, Group 3). The difference in effect on EST produced by the 10 mg dose and by the 0.5 and 3 mg doses of ACTH appears to be due in large measure to the weight loss

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4. Davenport, V. D., and Davenport, H. W., *J. Nutrition,* 1948, v36, 139.

5. Davenport, V. D., *Fed. Proc.,* 1950, v9, 28.

§ Unilateral nephrectomy and sodium chloride were employed in the present investigation in connection with parallel pathological studies on the influence of ACTH and cortisone on the toxic effect of DCA on tissues.

in animals injected with the inordinately large dose of ACTH. However, no final conclusion can be reached until the effects of these various doses of ACTH are compared under experimental conditions in which body weight changes are rigidly controlled.

Cortisone alone in a dose of 2 mg daily produced a marked reduction in the EST (Group 10). However, a marked reduction in body weight occurred, and the final evaluation of the experimental data, which suggest an increased brain excitability after cortisone, must therefore await additional studies with smaller doses of the steroid and with adequate control of the factor of body weight change. Baehr and Soffer(6) discontinued cortisone treatment in a young woman with lupus erythematosis because of the occurrence of repeated epileptiform convulsive seizures.

The effect of DCA on EST was effectively antagonized throughout the entire experiment by the daily administration of 2 mg of cortisone. The weight loss in the DCA-cortisone group was actually greater than that in the group given cortisone alone. However, despite the tendency of weight loss to decrease EST, the threshold of the animals on the combined steroid treatment was significantly higher than that of the animals given cortisone alone. A dose of 0.1 mg of cortisone counteracted the action of DCA during the first 20 days of the experiment. However, an appreciable, steady rise subsequently occurred. It is possible, although unlikely, that cortisone at this dose level acts similarly to the 0.5 and 3 mg doses of ACTH in inducing a secondary rise in threshold. It is noteworthy that the increase in EST induced by the 0.5 and 3 mg doses of ACTH started earlier than that induced by 0.1 mg of cortisone. In order to clarify this aspect of the problem and to explore the possible use of the cortisone-DCA antagonism as a method of assay for cortisone-like substances, additional experiments are being conducted in which doses of cortisone both smaller than 0.1 mg and intermediate between 0.1 and 2 mg are employed.

The concept has been developed in this laboratory(2,7-9) that administration of DCA induces a state of steroid hormone imbalance characterized by an excess of exogenous DCA and a deficiency of endogenous 11,17-oxysteroids (cortisone-like steroids); the deficiency is considered to be caused by (a) inactivity of the adrenal cortex as a result of inhibition of adenohypophyseal adrenocorticotrophic activity by DCA, and (b) antagonism between DCA and the 11,17-oxysteroids at their loci of action in tissues. The results presented here give additional experimental support to this concept. In a previous study(2) it was demonstrated that ACTH and ACE antagonize the action of DCA in decreasing brain excitability. The present experiments confirm the previous findings in regard to ACTH and in addition demonstrate that cortisone also antagonizes the EST-elevating property of DCA.

Summary. Rats treated with ACTH alone, in a dose of 1 mg thrice daily, initially showed a slight decrease in EST followed by a return to normal and subsequently a slight increase in threshold. ACTH in doses ranging from 0.001 mg to 10 mg effectively antagonized the action of DCA on the EST. Beyond the 12th day of hormone treatment, 0.001, 0.01, 0.05 and 10 mg of ACTH maintained the EST of DCA-treated rats at a normal or slightly less than normal level. Intermediate doses (0.5 and 3 mg) produced a secondary rise beginning about the 12th day of the experiment. Nevertheless, the final EST values in animals treated with 0.5 and 3 mg of ACTH were significantly less than in rats given DCA alone. Cortisone alone, in a dose of 1 mg twice daily, markedly reduced the EST of rats. This dose of cortisone given in combination with DCA resulted in an EST which was lower than normal but higher than that for animals treated with cortisone alone. Cortisone in a dose of 0.1 mg daily was able to counteract the EST-

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elevating property of DCA for the first 22 days of the experiment. Thereafter the EST increased to a final value intermediate between normal and that of rats treated with DCA alone.

The authors express their appreciation to Dr. Louis S. Goodman for his interest and help during the course of this study.

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Alterations in Long Bones of Young Albino Rats Induced by Dihydrotachysterol. (Hytakerol).* (18212)

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In the stages of transformation of ergosterol by ultra-violet radiation into a substance with antirachitic potency, Holtz and Schreiber(2) had determined the presence of two different factors, the antirachitic factor and a separate intermediate factor which they called the "Kalcinosefactor." A year later Windaus(3) reported the isolation of a toxic substance, tachysterol which he thought was a contaminant in highly effective antirachitic agents. Animal experimentation indicated that the dihydro-derivative of tachysterol had marked hypercalcemic effects, which suggested its use as a therapeutic agent in parathyroprivia (4,5). This drug was first used clinically by Holtz and its nature exhaustively restudied by von Werder(6). Albright and Ellsworth (7) had considered the action of parathormone was to raise the level of urinary phosphate excretion, and Albright(8) later demonstrated that the actions of parathormone, vitamin D and dihydrotachysterol were similar but that the action of the latter was more nearly like that of parathormone because it tended to increase urinary phosphorus excretion in contrast to that of vitamin D which increased intestinal absorption of calcium.

The present investigation was undertaken to determine the nature of changes occurring in growing bone resulting from toxic dosage of dihydrotachysterol and to compare these with changes reported, using other factors regulating bone architecture. The only such reference in English that we have found is a statement by McLean(9) that the lesions resulting from administration of vitamin D and dihydrotachysterol were similar and that no fibrous infiltration was observed as seen in hyperparathyroidism.

Methods. A group of 9 rats (A) 1 month old, were given daily subcutaneous injections over a period of from 30 to 35 days, total dosage varying from 3.75 mg to 5.5 mg of the crystalline dehydrotachysterol. Two litter mates were given daily injections of sesame oil and 2 others served as untreated controls (Table I). In 2 rats (Group B), 2 months old, the drug was given daily by stomach tube over a period of 10 days, total dosage being 1.03 mg and 1.16 mg of crystalline product. Again 2 litter mates were given an equivalent amount of sesame oil over the same

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* Some of these experiments were presented by title at the American Association of Anatomists, April 5, 1950(1).

1. Latta, J. S., and Tristan, T. A., *Anat. Rec.*, 1950, v106, 279.

2. Windaus, A., *Proc. Roy. Soc. London.*, Series B., 1931, v108, 568.

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7. Albright, F., and Ellsworth, R., *J. Clin. Invest.*, 1929, v7, 183.

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9. McLean, F. C., *J.A.M.A.*, 1941, v117, 609.

TABLE I. Drug and Oil Administered by Subcutaneous Injection. All animals 1 month old.

Animal	Length of exp. in days	Treatment	Total dosage
A 1 ♀	35	0	Control
2	35	0	,,
3	35	Sesame oil 3 cc	,,
4	35	4.4	,,
5	35	Dihydrotachysterol	3.75 mg cryst.
6 ♂	35	,,	4.375
7 ♀	30	,,	5.5
8	30	,,	3.75
9 ♂	30	,,	4.5
10	30	,,	4.375
11	30	,,	4.375
12	30	,,	4.375
13	30	,,	4.375

TABLE II. Drug and Oil Administered by Stomach Tube. All animals 2 months old. Length of experiment for each animal, 10 days.

Animal	Treatment	Total dosage
B 1 ♀	0	Control
2 ♂	0	,,
3	Sesame oil 10.3 cc	,,
4 ♀	23.3	,,
5	Dihydrotachysterol	1.03 mg cryst.
6 ♂	,,	1.16

TABLE III. Drug and Oil Administered by Stomach Tube. All animals 2 months old.

Animal	Length of exp. in days	Treatment	Total dosage
C 1 ♀	7	0	Control
2	14	0	,,
3	21	0	,,
4	7	Sesame oil 3.1 cc	,,
5	14	7.2	,,
6	21	11.1	,,
7	7	Dihydrotachysterol	.31 mg cryst.
8 ♂	7	,,	.42
9 ♀	14	,,	.81
10 ♂	14	,,	1.15
11 ♀	21	,,	1.67
12 ♂	21	,,	1.68
13	28	,,	2.64
14	42	,, 28 days 14 days recovery	2.55

period and 2 others remained as untreated controls (Table II).

A third group of 8 animals (C) were given the drug every second to fourth day by stomach tube over a period of from 1 to 4 weeks, total dosage varying from .31 mg to 2.645 mg of the crystalline product. Controls for this group were similar to those in Groups A and B (Table III).

During the experimental period the animals were fed Rockland Rat Diet supplemented by corn, carrots and green vegetables. Weight charts on individual animals of Group A showed no significant variations between controls and treated animals. In Groups B and C, however, with oral administration the treated animals failed to gain and in most cases, lost weight after 6 to 10 days of treatment. This tendency was observed also in animals given only sesame oil orally. At the end of the experimental period the animals were killed, the long bones removed, fixed in Zenker-formol solution, decalcified, sectioned and stained in routine manner, Masson's trichrome stain being most used. Samples of liver, lung and kidney were also obtained for reference.

Experimental results. Controls. Examination of histological preparations of femurs from untreated controls and those either injected or fed sesame oil gave findings in all cases consistent with those of all rats in the various age groups. In all cases the epiphyseal cartilages were proliferative with regular zones of provisional calcification on the diaphyseal side. Trabeculae in the metaphyses were regularly arranged and covered with a row of osteoblasts. The compact bone of the shaft was regularly and evenly formed with no irregularities on either periosteal or endosteal surfaces.

Group A. In all the animals of Group A, however, subjected to daily subcutaneous injections of dihydrotachysterol, the epiphyseal cartilages were somewhat wider than normal with no apparent alteration in the cartilage matrix. The diaphyseal border was very irregular, some columns of hypertrophic degenerating cartilage cells extending deeply into the metaphysis. On the epiphyseal side of the cartilage the cell groups were somewhat irregular also (Fig. 1). The most marked changes observed in the treated animals of this group were found in the compact bone of the shafts. The thickness of the bone was considerably decreased. Numerous cystic spaces and channels had perforated this compact bone (Fig. 2), in some cases offering

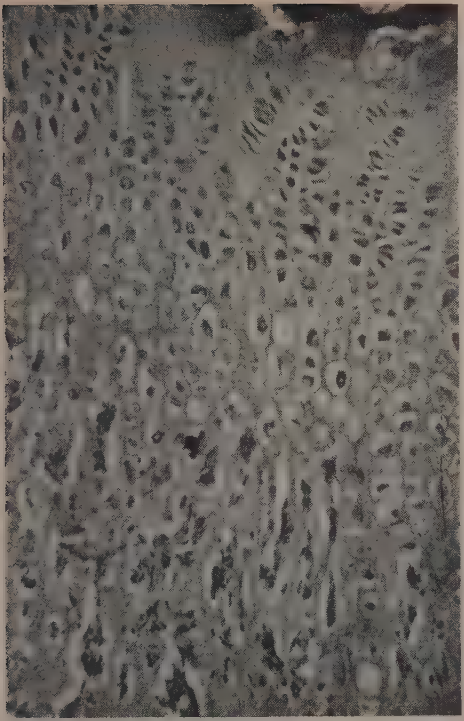

FIG. 1.
Femur, rat A6, treated subcutaneously for 35 days, total dosage 5.5 mg cryst. dihydrotachysterol, showing distortion of cell columns in the epiphyseal cartilage and irregular lacy trabeculae in the metaphysis. ×165.

wide communication between the periosteum and the marrow cavity. Closer examination showed these spaces to be quite variable in size and shape, but limited largely to the inner two-thirds of the bone. In some instances these spaces were filled with hemopoietic tissue. Most of the spaces contained a very vascular connective tissue with numerous fibrocytes with large pale nuclei and slender processes. Around the periphery of some of these cavities particularly the most extensive ones, occasional multinucleated osteoclasts were encountered (Fig. 3). Histological examination of samples of liver, lung and kidney and parathyroid from animals treated with the drug revealed no evidences of calcification, necrosis or other pathological alteration.

Group B. In the 2 animals given dihydrotachysterol daily for 9 and 10 days respectively despite evident disturbance as indicated by weight loss there was little evidence of an alteration in the bony structure. Epiphyseal cartilages, and epiphyseal and metaphyseal trabecular bone were quite similar to that even in oil treated and untreated controls. However, in the compact bone of the shaft the endosteal surfaces were characterized by semicircular depressions. In some places this surface had thin inner layer of more basically stained bone, evidently of recent deposition.

Sections of livers of these animals revealed in one case (a female) a marked fatty change in the liver cells in the central portion of the lobules indicated by the presence of one or more large cytoplasmic vacuoles. Cells in periportal areas exhibited cloudy swelling. A few apparent cases of brightly stained precipi-

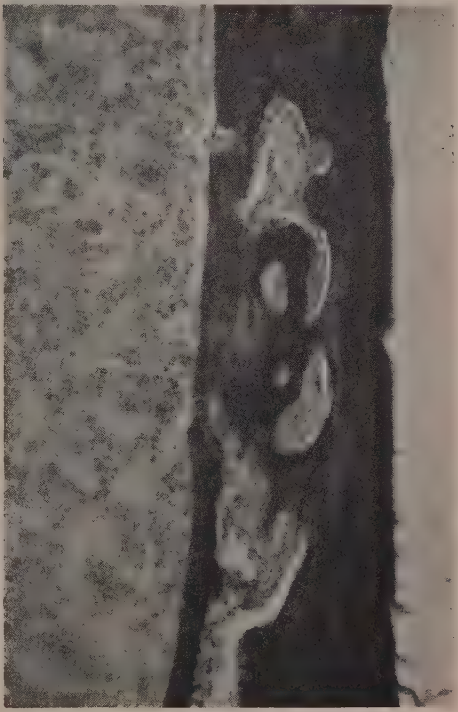

FIG. 2.
Femur, rat A5, treated subcutaneously for 35 days, total dosage 3.75 mg cryst. dihydrotachysterol, showing extensive channels and cystic spaces in the cortex of the shaft resulting from osteolysis. ×165.

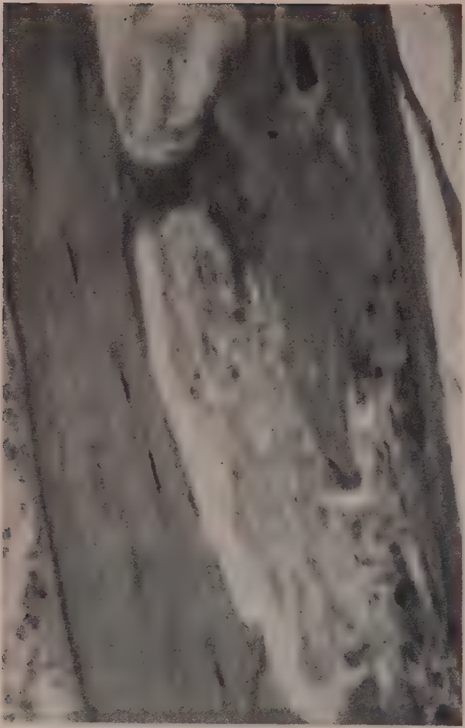

FIG. 3.
Detail of a portion of the cortex of the femoral shaft of rat A6, (same as in Fig. 1) illustrating infiltration of one of the cavities by cellular fibrous tissue. Note the enlargement of the bone lacunae. ×420.

tated material were found in and around nephric tubules, but no areas of definite calcification were seen.

Group C. In this group the sesame oil and dihydrotachysterol were fed by stomach tube every second, third or fourth day, over periods of from 1 to 4 weeks, thus varying the total dosage considerably and allowing greater total dosage without lethal effects. In those animals treated for 1 or 2 weeks, with relatively low total dosage, alterations similar to those described in Group B were encountered in the long bones. As treatment was prolonged 3 or 4 weeks and total dosage correspondingly increased, marked changes were found resembling but reaching greater severity than those described for Group A, injected subcutaneously.

The most obvious changes were a marked decrease in thickness of the compact bone, extensive widening of the existing Havervian canals, and great irregularity of, the endosteal surfaces (Fig. 4). Numerous spaces in bone had become invaded either by fibro-vascular or actively hemopoietic tissue. Some of these extended almost or quite to the periosteal surface (4 weeks) so making it difficult to conceive of the bone as effective in weight bearing, and making histological preparation difficult (Fig. 5). The epiphyseal cartilages were most irregular in width, some groups of swollen cartilage cells being found deep in the metaphyses. The regularity of the cell columns was again much distorted which was to be associated with equal distortion of metaphyseal bone which consisted of a lacy net-

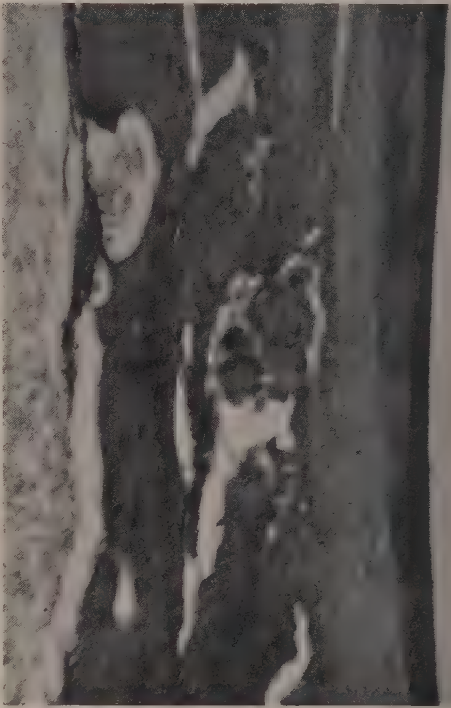

FIG. 4.
Cortex of the femoral shaft of rat C12, with total dosage of 1.68 mg cryst. dihydrotachysterol given by stomach tube over a 3 weeks period. Extensive breakdown of the lamellar pattern, cavitation, and isolation of bone fragments are noted. ×323.

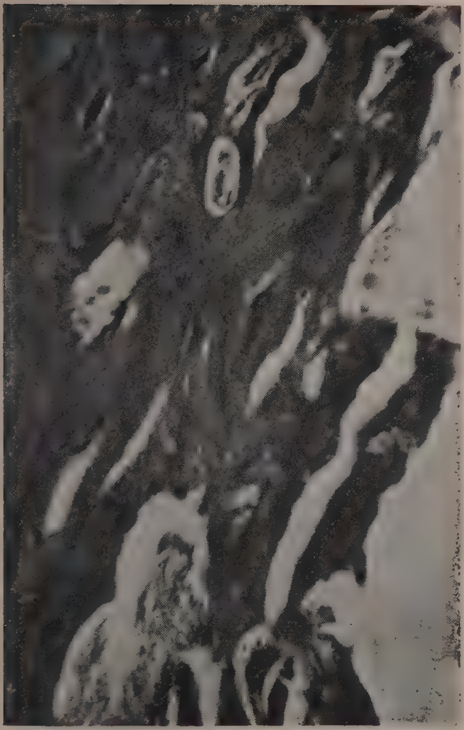

FIG. 5.
Cortex of the femoral shaft of rat C13 treated by stomach tube, over a 4 weeks period, with total dosage of 3,645 mg cryst. drug. Very extensive breakdown, with channeling and cavitation throughout the inner portion of shaft, with some infiltration by marrow tissue. ×323.

work of slender trabeculae. This same distortion was also apparent in the spongy bone of the epiphyses. These changes were of course most marked in animals treated over a 4 week period. In the one animal so treated and allowed 2 weeks for recovery there was no evidence of a recovery in the skeletal architecture.

In some animals following 4 weeks of treatment by feeding particularly the rat allowed a 2 weeks recovery period. The histological structure of the liver and kidneys was considerably altered. After 4 weeks liver cells placed centrolobularly were swollen, granular and vacuolated, many with pycnotic nuclei. After the same treatment and 2 weeks recovery this change, resembling that seen with heavy glycogen deposition, was seen in practically all liver cells.

In all cases after the first week of treatment tubular casts and stromal deposits of heavily stained, apparently precipitated material, were present increasingly as the time and total dosage increased. Tubular block and resultant dilation of proximal tubules and Bowman's capsule became very prominent in those animals receiving greater total dosage. Epithelial degeneration, sluff and underlying regeneration were frequently seen in proximal (?) tubules in the case in which 2 weeks recovery time was allowed. In scattered areas definite calcified deposits were found.

Discussion. The abundant evidence presented here of bone resorption in young rats resulting from chronic dosage with dihydrotachysterol is in partial agreement with findings of Lenel(10) on chronically treated rats. We found considerably less evidence of regenerative activity expressed in newly formed osteoid tissue. The bones of our experimental animals, maintained on a completely adequate diet, did not exhibit any evidence of bone necrosis as shown by Lenel. The series of channels and cystic spaces extending principally into hemopoietic marrow or, at first with cellular fibrous tissue, suggesting lesions of hyperparathyroidism. With the high total dosage given over a 4 week period this resorption of bone salts reached marked proportions. The failure to find evidence of reparative growth in the individual allowed 2 weeks for recovery indicates that the resorptive effect of the drug is cumulative and long lasting and does not support Lenel's findings of an osteosclerotic phase, over long experimental periods.

Our reported findings of distortion occurring in the epiphyseal cartilage and irregularities in the resorption process in the metaphyses of the long bones during and following treatment have not previously been reported. The experiments reported by Eger and Titze (11) were not comparable to those we have presented but do not support our findings of bone resorption, because of difference in dos-

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10. Lenel, R., *Frankf. Z. Path.*, 1941, v56, 31.

11. Eger, W., and Titze, G., *Klin. Wochensch.*, 1942, v21, 859.

age. The increasing incidence of calcium (?) casts in the convoluted tubules and deposits in interstitial tissue of the kidney as total dosage increased indicates a direct relationship between the amount of dihydrotachysterol present, the amount of calcium removed from bony tissues, and its excretion by the kidneys.

The similarity between the lesions in cortical bone we have demonstrated and those experimentally produced by overdosage with commercial vitamin D products, as viosterol (*e.g.* Jones and Robson)(12), leads to the suggestion that the osteolytic effects encountered in the latter case were induced by the presence of dihydrotachysterol as a contaminant. It is interesting to note that similar lesions in cortical bone were demonstrated in guinea pigs depleted of vitamin C by Silberberg and Silberberg(13), probably induced in that instance by disappearance of the organic matrix.

It is quite possible that blocks resulting from calculi and interstitial calcification and subsequent dilation of damaged renal tubules in the more advanced stages of the treatment were of sufficient degree to have secondarily significantly altered the capacity of the kidney to excrete calcium and reabsorb phosphates.

Results obtained from administration of the drug by injection and by forced feeding indicate its greater absorption and effectiveness by the latter route as shown by the greater degree of bone resorption in animals of group C as compared with those of group A, treated over similar time periods.

Summary. Groups of young albino rats were treated either by repeated subcutaneous injections or by repeated forced feedings with dihydrotachysterol (Hytakerol) over periods ranging from 7 to 30 days, total dosage of the drug varying from .31 to 5.5 mg. The histological alterations in the long bones resulting from this treatment have been described. Significant changes were induced with relatively lower dosage when the drug was given by stomach tube but with either method were in direct proportion to the total dosage and duration of the experiment. The most striking changes consisted of the appearance of extensive channels, wide passageways and cystic cavities extending varying distances from the endosteal toward the periosteal surfaces of the cortex of the long bones. After prolonged oral administration most of the shaft was reduced to a trabecular meshwork. Some of these spaces were occupied by highly vascular hemopoietic tissue, with some infiltration of fibroblastic cellular tissue. Infrequently at the distal ends of these spaces, large multinucleated osteoclasts were found. With chronic oral treatment of a 10-day period only minor irregularities were encountered on the endosteal surface and a thin layer of osteoid tissue, apparently recently deposited, was present.

Although with lower dosage, no changes were found either in the metaphyseal bone or the epiphyseal cartilage, with great dosages and longer time intervals several variations were noted in the cartilage, and the metaphyseal bony trabeculae were very slender and distorted. We considered all of these changes to be the result of direct action of dihydrotachysterol causing a removal of calcium from bone. The appearance in late stages of renal tubular calculi, blockage, distention and cellular degeneration in proximal tubules were presumed to be the result of hypercalcemia secondary to the osteolysis caused by the drug.

The dihydrotachysterol (Hytakerol) used in the experiments was furnished through the courtesy of Dr. A. Scribner of Winthrop-Stearns, Inc.

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12. Jones, J. H., and Robson, G. M., *Am. J. Physiol.*, 1933, v103, 338.

13. Silberberg, M., and Silberberg, R., *Anat. Rec.*, 1948, v102, 141.

Serological Reactions of L Type Cultures Isolated from Proteus.*† (18213)

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Freshly isolated Proteus cultures when transferred to soft horse serum agar plates containing high concentrations of penicillin usually produce L type colonies abundantly(1). These correspond in appearance and morphology to the Ll isolated from *Streptobacillus moniliformis* by Klieneberger. On appropriate media penicillin induces a similar transformation in various species of Gram negative and Gram positive bacilli(2). However, the development of L type colonies has been observed under varying conditions in several species in the absence of penicillin(3). Two types of colonies develop on penicillin plates inoculated with Proteus. One type grows to a larger size, does not require animal serum for development and reproduces the usual bacilli when transferred to penicillin-free media. These colonies have been designated as 3B L type colonies. On soft horse serum agar plates containing penicillin, many tiny colonies also develop among the 3B colonies. These colonies can be subcultured only on media containing animal serum and have been designated as 3A L type colonies. These colonies also reproduce the usual bacillary forms of Proteus on penicillin-free media. In contrast to the large 3B colonies, this requires at least 24 hours and may be delayed for several weeks if the cultures have been propagated for a long period of time in the presence of penicillin. There is no essential difference in morphology between the organisms in the 3A and the 3B L type colonies. When the 3B colonies are transferred to soft horse serum agar containing penicillin, abundant small 3A colonies develop, indicating the close relationship between the 3A and 3B L type colonies. The conditions under which 3A and 3B L type colonies develop, their similarity to the Ll of *Streptobacillus moniliformis* and their properties have been described elsewhere(1). The consistent reappearance of the usual bacillary forms of Proteus from these colonies, even after long cultivation on penicillin plates, leaves no doubt that the L type colonies are produced by Proteus growing in a changed form. This conclusion is supported also by the observation that the L type colonies grow out from the bacilli. The bacilli, as in *Streptobacillus monoliformis* and Bacteroides, first swell into large bodies from which the L forms grow. Further evidence for the identity of the bacillary and L forms is their serological similarity. Data concerning this will be presented in this paper.

The serological study of the L type cultures was undertaken primarily to obtain additional evidence of their genetic relationship to the bacilli. A more thorough study of the antigenic structure of the L forms may provide information as to the nature of the L transformation of bacteria and would therefore be of great value. The technical difficulties involved made it necessary to restrict the present work to the original purpose.

Cultures of the small 3A L type colonies were used for the serological studies. These cultures are easier to keep growing than the large 3B L type colonies. Furthermore, the differences between the 3A and the bacillary cultures are more marked than between the 3B and the bacillary cultures. Thus, serological similarity between the 3A L type cultures and the usual bacillary cultures is more significant. The 3A cultures when they are adapted to the medium grow abundantly on

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† This is publication No. 116 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

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3. Dienes, L., Weinberger, H. J. and Madoff, S., *J. Bact.*, 1950, v59, 755.

TABLE I. Agglutination Reactions of *Proteus bacilli* and of Their L Forms.

Rabbit antisera	Antigens													
	#3 Bac								#3 L					
	1:40	80	160	320	640	1280	2560	5120	1:20	40	80	160	320	640
3 Bac	3+	3+	3+	3+	3+	+	0	0	+	2+	3+	3+	2+	+
3 L	3+	2+	2+	2+	+	±	±	0	3+	3+	2+	+	0	0
14 Bac	0	0	0	0	0	0	0	0	0	±	0	0	0	0
14 L	+	+	±	0	0	0	0	0	0	0	0	0	0	0
52 Bac	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52 L	2+	+	0	0	0	0	0	0	0	0	0	0	0	0
	#14 Bac								#14 L					
3 Bac	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
3 L	3+	3+	3+	3+	2+	2+	2+	0	0	0	0	0	0	0
14 Bac	3+	3+	3+	3+	2+	+	0	—	2+	2+	2+	+	0	0
14 L	3+	3+	2+	2+	2+	2+	2+	—	+	±	0	0	0	0
52 Bac	3+	3+	2+	+	0	0	0	0	0	0	0	0	0	0
52 L	+	+	±	0	0	0	0	—	0	0	0	0	0	0
	#52 Bac								#52 L					
3 Bac	2+	±	0	0	0	0	—	—	0	0	0	0	0	0
3 L	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14 Bac	3+	3+	3+	+	0	0	—	—	0	0	0	0	0	0
14 L	±	0	0	0	0	0	0	0	0	0	0	0	0	0
52 Bac	3+	3+	3+	2+	2+	+	—	—	0*	0*	0	0	0	0
52 L	±	0	0	0	0	0	0	0	3+	3+	3+	3+	2+	+

* Slight agglutination with 0 serum (obtained with boiled cultures).

serum agar plates. In 5 to 7 days they produce a thick firm film in heavily inoculated areas. The individual colonies are hard and rubbery, 1-2 mm in size. The culture is firmly embedded in the medium and cannot be separated from it. For the inoculation of the rabbits the L cultures were grown on soft agar containing rabbit serum and 1600 units of penicillin per ml. The surface of the agar containing the cultures was scraped off, emulsified in saline and injected intraperitoneally. Three L type cultures isolated from our Proteus strains 3, 14 and 52 were used for this purpose. These strains had been used previously for other studies. Other rabbits were treated by intravenous injection of the bacilli, some with fresh living organisms and some with organisms killed by boiling. The rabbits were injected on 2 consecutive days of each week for 6 weeks. The agglutination reactions of the bacillary antigen with the sera obtained after 4 injections of the living cultures are recorded in Table I. The strain specificity of these sera was more marked than that of sera obtained after further inoculation. With the L antigens stronger reactions were obtained both with the bacillary and L sera after further treatment of the rabbits and the reactions with these sera are recorded in the table.

The most successful method to obtain an emulsion of the L cultures for the agglutination test was the following: Squares of agar with the well-grown cultures were cut out and inverted with the colonies downward in a petri dish containing 1-2 ml of salt solution. By rubbing the agar blocks on the dish a portion of the colonies was emulsified. After sedimentation of the larger particles, the emulsion was stable and gave specific agglutination reactions. The emulsion did not consist of single organisms but of small clumps of granules and of large forms of various sizes. Compared with the bacillary antigens the agglutination titre of the L antigens remained low and agglutination occurred in the form of loose flocci. In positive tests the turbidity of the L antigens markedly increased with higher concentrations of the sera. In these respects the reaction resembled the precipitin test. A clear filtrate of the emulsion and the extract of the agar medium on which the L type culture was grown gave a strong precipitin test with specific sera. The L forms of

Salmonella gave the same type of agglutination as the Proteus L, although no precipitation was obtained with extracts of Salmonella L type cultures(4).

The differences in the reactions of the 3 strains were marked both *in vivo* and *in vitro*. The bacillary and L antigens of Strain 3 reacted well with the antisera obtained with both and reacted only slightly or not at all with any of the other sera. In previous experiments this strain had presented the most marked strain antagonism against other Proteus strains. The 3 L serum agglutinated the bacillary antigen of strain 14 but not the L emulsion. In strain 14 also the common reactions between the bacillary and L antigens and antisera were marked. The 14 L antigen was agglutinated only by the strain specific sera; the bacillary antigen was agglutinated somewhat by all sera. In strain 52 there was hardly any common reaction between the bacilli and the L forms; the L sera agglutinated the bacillary antigens of strains 3 and 14 slightly more than their own. The Proteus bacillary and L antigens were tested with antisera against *Salmonella typhosa* and *S. typhimurium* and the Salmonella bacillary and L antigens were tested with Proteus antisera. No overlapping of reactions was observed between the L forms of Proteus and Salmonella.

The L forms of Proteus apparently show a marked strain specificity in the agglutination test. In 2 strains they reacted only with sera produced by themselves and by the corresponding bacilli. However, the sera produced by the L forms have a wider range and react more often with bacillary antigens of nonspecific strains. This difference between the *in vivo* and *in vitro* specificity may be only an apparent one, a consequence of the low sensitivity of the L cultures in the agglutination test.

The serological reactions of the L forms of *Streptobacillus moniliformis, Salmonella typhosa* and *Salmonella typhimurium* have been studied and found to be very similar to those of the corresponding bacilli(5,6). The results with Proteus strains 3 and 14 correspond to these observations, but strain 52 is apparently an exception. The bacteria regained from the L forms were examined and found to be unchanged in their serological properties. In some experiments L forms isolated from different strains were grown in mixed culture; no change was observed in the serological properties of the bacilli regained from these cultures.

Agglutinin absorption tests were conducted with bacillary and L sera of strains 3 and 52. In each case both bacillary and L emulsions were used for absorption of the antibodies. Three cc of the serum diluted 1:10 was mixed with a loopful of bacillary mass taken from an agar culture and then placed in the refrigerator for 1 hour. The serum was then centrifugalized and the supernatant was again mixed with a loopful of culture mass and placed in the refrigerator for 1 hour. After centrifugalization the supernatant was removed and was used for the agglutination test. The absorption of the sera with the L cultures was performed in a similar manner. For each absorption half of the culture scraped from a well-grown agar plate was mixed with the serum. The amount of L culture added to the serum was probably sufficient but it could not be emulsified as well as the bacilli.

The low titre of the sera against the L antigen makes the interpretation of the results somewhat uncertain. The 52 sera which had not shown any common reaction between the bacilli and L forms were not noticeably weakened by absorption with the heterologous antigen. The bacillary emulsion of strain 3 absorbed the agglutinins both from the bacillary and L sera. The L emulsion absorbed the agglutinins from the L sera against both the bacillary and L antigens but did not weaken the reaction of the bacilli with the bacillary sera. According to these limited observations the bacillary and L forms of strain 52 both contain antigens which are not active in the other either in the agglutination test *in vitro* or in the immunization of the rab-

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4. Weinberger, H. J., Madoff, S., and Dienes, L., *J. Bact.*, 1950, v59, 765.

5. Heilman, F. R., *J. Infect. Dis.*, 1941, v69, 32.

6. Dawson, M. H., and Hobby, G. L., Third International Congress for Microbiology, *Abstracts of Communications,* 1939, p. 21.

TABLE II. Agglutinin Absorption Tests with Bacillary and L Immune Sera (Proteus Strain 3)

Rabbit antisera	Bacillary antigen					L antigen				
	1:20	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320
	Agglutination Before Absorption.									
3 Bac	+	2+	3+	3+	2+*	+	2+	3+	3+	2+
3 L	3+	3+	3+	2+	2+†	3+	3+	3+	2+	0
	Agglutination after absorption with bacilli									
3 Bac	0	±	±	0	0	+	±	±	0	0
3 L	0	0	0	0	0	0	0	0	0	0
	Agglutination after absorption with L									
3 Bac	2+	3+	2+	2+	2+	±	±	±	0	0
3 L	±	0	0	0	0	0	0	0	0	0

* The titer of this serum was 1:5120.
† ” ” ” ” ” ” 1:2560.

bits. The L forms of strain 3 which gave a marked common reaction with the bacilli seem to lack certain antigens which are present in the bacilli. The agglutinin absorption experiment conducted with Proteus strain 3 is recorded in Table II.

Summary. The serological properties of 3 Proteus strains and the L forms isolated from them have been studied. The L antigens were agglutinated both by the bacillary and L immune rabbit sera in a relatively low titre and agglutination occurred in large loose flocci. Clear filtrates of L emulsions gave a good precipitation. The reactions of 2 L type cultures were strain specific. A marked common reaction was present between the bacillary and L forms of 2 strains both in the production of antibodies and in the *in vitro* tests. There was only a slight overlapping in the reactions of the L forms and of the bacilli in the third strain. The similarity of the serological reactions of the bacillary and L forms of 2 Proteus strains is further evidence for the genetical identity of the L forms and the bacilli. The nature of the serological dissimilarity between the bacillary and L forms of one strain, apparent both in *in vitro* tests and in antibody production, can not be interpreted at present.

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Isolation of L Type Cultures from Clostridia.[*][†] (18214)

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Under certain conditions many Gram negative bacilli grow in very small soft pleomorphic forms which are usually designated as pleuropneumonia-like or L forms. The development of similar forms has been observed in the cultures of a few large aerobic Gram positive bacilli probably belonging to the genus Bacillus(1). The isolation of L forms from 2 strains of Clostridium is the subject of this paper. The most efficient method for the isolation of L forms is exposure of the bacilli to penicillin on agar plates. Many Gram negative bacilli produce L type colonies abundantly if they are inoculated on soft horse serum agar plates containing either 400 or 1600 units of penicillin

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1. Dienes, L., Proc. Soc. Exp. Biol. and Med., 1949, v71, 30.

per ml of the medium. L forms were isolated from Gram positive aerobic bacilli also on similar plates originally free of penicillin but on which 1000 units of penicillin was deposited in a small trough cut out of the agar. Several Clostridia strains (*Cl. welchii, tetani, botulinum* and *sporogenes*) were tested with both these techniques. These were old laboratory strains from the culture collection of the Bacteriology Department at Harvard Medical School. The plates were incubated anaerobically with Fortner's biological method.

None of the strains showed any growth or changed its morphology on the penicillin plates. On penicillin-free plates growth of the bacilli was inhibited in a large area around the penicillin trough. Except in the area immediately surrounding the trough the bacilli started to multiply, but the multiplication stopped and many bacilli swelled to large bodies or disintegrated into small granules when they were reached by the diffusing penicillin. Outside the area of inhibition normal bacterial growth developed and, in the case of *Cl. tetani,* covered the plates as a spreader. No further growth developed in the area of inhibition on the plates inoculated with *Cl. welchii, botulinum* or *sporogenes.* On the plates inoculated with *Cl. tetani,* L type colonies started to grow from the large bodies after 24 hours' incubation and within a few days developed to macroscopically visible size. These colonies grew without difficulty in subcultures on similar media either with or without penicillin.

The macroscopic appearance and morphology of the L type cultures of *Cl. tetani* are similar to those of the third Gram positive aerobic bacillus previously described(1) and of the L type cultures of Bacteroides(2). The photographs illustrating these two could be used to illustrate the L type cultures of *Cl. tetani* as well. These, like the L type cultures isolated from the aerobic Gram positive bacilli, grew better on media without animal serum. No growth was obtained in broth, and the bacillary form of tetanus was not recovered from the L type cultures. No tetanus developed in several mice injected with the L type cultures.

It is possible that most of the Clostridia studied gave negative results because they were old stock strains. It has already been noted that stock strains of Gram negative bacilli also produce L forms less readily. A slight L type growth starting from the large bodies was observed in another stock strain of *Cl. welchii* examined in a similar manner, and L type colonies were isolated without difficulty from an unidentified Clostridium recently cultivated in this laboratory. The observations made with this strain are of interest because the L forms developed under markedly different conditions from those observed with *Cl. tetani.*

This Clostridium strain (2124) was obtained from an apparently normal lung at post mortem examination. No sign of gas bacillus infection was present. The bacillus was strictly anaerobic; it coagulated milk and produced gas abundantly in milk and in meat tubes in 24 hours. Central spores developed in moderate numbers. Immediately after isolation the colonies on agar plates were large, mucoid and with even contours. After several transfers a spreading growth started from the colonies if the plates were incubated for several days. Injection of 0.1 ml of broth culture into guinea pigs produced extensive liquefaction of the subcutaneous tissue and death in 24 hours. These characteristics identify the strain as a Clostridium; further identification is not important, because the phenomena described are more characteristic of the individual strain than of the species and they could be observed only for a short period following isolation of the strain. Clostridium strain 2124 was studied first with the same technique as described with the other Clostridia. The bacilli swelled to large bodies in the neighborhood of the penicillin trough, but no L type growth developed. The culture was slightly pleomorphic on blood agar plates and a few large bodies developed without exposing the cultures to penicillin. In a 2-day-old culture with abundant growth a few colonies were spread again over the previously uninoculated areas of the plate. Growth was

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2. Dienes, L. and Smith, W. E., *J. Bact.*, 1944, v48, 125.

inhibited in a wide area around the primary culture. The bacilli inoculated in that area swelled to large bodies and many L type colonies started to develop from them. They grew without difficulty in subcultures either in the presence or absence of penicillin. In this strain the growth of L type colonies was not induced by penicillin, but by exposure of the bacilli to their own metabolic products. Accumulation of metabolic products is probably responsible for the spontaneous development of L type colonies in Gram negative bacilli. This was especially apparent in a strain of Bacteroides in the cultures of which accumulation of metabolic products and the presence of penicillin produced a similar pleomorphism and the development of L forms(2). It is possible that L type cultures could be obtained from our strain 2124 by penicillin also, if the conditions under which the bacilli exposed to it were sufficiently varied.

As previously mentioned, the development of L type cultures in strain 2124 was observed only for a short period of time following isolation. Later the pleomorphism of the strain disappeared and the spreading growth made impossible the use of the technique which had given positive results initially. The L forms isolated from this strain were similar in appearance and morphology to the L forms of *Cl. tetani.* They grew well on nutrient agar without animal serum; they did not grow in broth and the bacilli were not regained from them. The L type colonies of both Clostridia strains studied continued to grow for several months if the medium was appropriate and if the colonies were well-spaced. The L type colonies of tetanus grew sometimes to a size of 6 mm spreading in star-like streamers under the surface of the agar. Some L type colonies of strain 2124 developed to a size of 10 mm or more during a period of 2 months. The colonies spread slowly both on and beneath the surface of the medium. The spreading edge of these large colonies consisted of small forms similar to those present in young colonies. The center of the colonies was autolyzed and only a few large bodies remained intact and could be stained in them.

The genus Clostridium has been added to the groups of bacteria in which L type cultures have been observed. Such cultures have been observed in several families of Gram negative bacilli(3), in the Neisserriae and in aerobic Gram positive spore-bearing bacilli. It is possible that the ability to grow in such forms is a general property of bacteria. The appearance and morphology of the L forms and the conditions under which they develop in the cultures are similar in all cases. There seems to be a marked difference in growth requirements between the L forms isolated from Gram negative and those from Gram positive bacilli. The former can be maintained in continuous growth only in media containing fresh animal protein, whereas the L forms of Gram positive bacilli grow better if fresh protein is absent.

Reversion of the L forms into the usual bacilli and the serological similarity of the bacilli and the L forms has been observed in several species of Gram negative bacilli (Proteus(4), Salmonella(3), *H. influenzae,* Bacteroides(2) and *Streptobacillus moniliformis*(5)). These observations leave hardly any doubt that the bacilli and the L forms are different growth forms of the same organism. This opinion is now held by all investigators working in this field(6). However, the nature and significance of the L forms is entirely unknown. The availability of these forms in many different groups of bacteria may provide an opportunity to get information in this matter.

Summary. L type cultures have been isolated from a strain of *Clostridium tetani* with the help of penicillin. Similar cultures were obtained from a freshly isolated unidentified Clostridium when the area surrounding a 2-day-old agar culture was reinoculated with the same strain. The metabolic products diffusing from old culture inhibited growth

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3. Weinberger, H. J., Madoff, S., and Dienes, L., *J. Bact.,* 1950, v59, 765.

4. Dienes, L., *J. Bact.,* 1949, v57, 529.

5. Brown, T. McP., and Nunemaker, T. C., *Bull. Johns Hopkins Hosp.,* 1942, v70, 201.

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and transformed the bacilli into L forms. The L type cultures obtained are similar in appearance and morphology to those isolated previously from a Gram positive spore-bearing bacillus. They differ from the L forms of Gram negative bacilli inasmuch as they grow better in the absence rather than the presence of animal serum and have a tendency to form large spreading colonies increasing in size for several months.

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The Storage of Methoxychlor in the Fat of the Rat. (18215)

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(Introduced by A. J. Lehman)
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Methoxychlor, p,p′ dimethoxy diphenyl trichloroethane has been shown to have a considerably lower order of toxicity than DDT(1,2). There is some indication, as in the case of certain isomers of benzene hexachloride(3), that a low order of toxicity may be associated with a reduced tendency for storage in the fat. A study of the storage of methoxychlor has therefore been made to see whether a similar correlation exists.

Method. Three groups of 24 weanling 21-day-old rats, equally divided as to sex were placed on diets containing methoxychlor at concentrations of 25, 100 and 500 p.p.m. After 4-, 9-, 13- and 18-week exposures, 2 males and 2 females from each group were sacrificed and abdominal fat analyzed for methoxychlor. Equal numbers of control animals were treated similarly. At the end of 18 weeks, exposure to methoxychlor was terminated and the survivors placed on control diet. Following 2 and 4 weeks on control diet the remaining animals were sacrificed and the tissues also analyzed for methoxychlor. The basic diet was a ground commercial rat biscuit with a fat content of 5%. Analysis for methoxychlor gave negative results. The experimental diets were prepared by mixing appropriate amounts of corn oil solution of methoxychlor with the ground rat biscuit. The methoxychlor used was the p,p′ isomer and was of technical grade. The experimental animals were kept in separate cages and food consumption and weight gains recorded weekly. The control animals were housed in groups, and their weights recorded at approximately four-week intervals. Tissues were prepared for analysis by successive extraction of the fat with ethyl ether. Methoxychlor was determined by a method developed in this laboratory(4). This method consists essentially of saponification of the fats by ethanolic KOH, with simultaneous conversion of the methoxychlor to the dehydrochloride. Separation of the dehydrochloride of methoxychlor from the saponified fats is effected by extraction with petroleum ether. The dehydrochloride is then nitrated by the standard Schechter procedure. Upon treatment with sodium methylate in benzene-methanol solution a stable red color is produced having an absorption maximum at 530 mμ. The sensitivity is sufficiently great to quantitate 5 μg of methoxychlor when contained in a maximum of 5 g of rat fat (1 p.p.m.).

Results. The incorporation of methoxychlor at concentrations of 100 and 500 p.p.m. in the diet produced a retardation of growth in both male and female rats. After 14

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2. Smith, M. I., Bauer, H., Stohlman, E. F., and Lillie, R. D., *J. Pharm. and Exp. Therap.*, 1946, v88, 359.

3. Davidow, B., Frawley, J. P., Fitzhugh, O. G., and Woodward, G., unpublished observations.

4. Prickett, C. S., Kunze, F. M., and Laug, E. P., *J. Assn. Off. Agr. Chemists*, in press.

TABLE I. Storage of Methoxychlor in the Perirenal Fat of the Rat.

P.P.M. methoxychlor in diet	P.P.M. methoxychlor* retained in the perirenal fat											
	During exposure								Cessation of exposure			
	4 wk		9 wk		13 wk		18 wk		2 wk		4 wk	
	M	F	M	F	M	F	M	F	M	F	M	F
0							0	0				
25	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	4	7	3	4	1	1	0	0	0	0
500	36	17	26	20	19	14	16	14	0	0	0	0

* Each figure represents the avg of 2 animals with the exception of the control (0.0 p.p.m. methoxychlor in diet) where average represents 8 animals.

weeks, retardation at the 100 p.p.m. level was slight, but definite; at the 500 p.p.m. level retardation was marked averaging 60 g for the males and 40 g for the females. No effect was noted at the 25 p.p.m. level. None of the animals, on the 100 and 500 p.p.m. diets, however, showed any toxic symptoms. This is consistent with unpublished observations(6) from this laboratory on adult animals fed diets containing as much as 1000 p.p.m. methoxychlor. Since the effect on growth was unexpected, the food consumption of the 500 p.p.m. group was examined. It was found that the animals on the 500 p.p.m. methoxychlor diet consumed on the average about 25% less food than the animals on the 25 p.p.m. level. Thus, lower acceptability of the high methoxychlor diet may be the explanation. Further evidence in this direction is the observation that a number of methoxychlor animals, upon being put on control diet rapidly resumed normal food consumption and weight within a period of several weeks.

Table I shows the results of analyses of perirenal fat for methoxychlor. It is evident that no storage of methoxychlor occurs at the 25 p.p.m. level, very slight at the 100 p.p.m. level, and although measurable, only moderate even at the 500 p.p.m. level. In contrast to DDT(5) there is no tendency for the female to store more methoxychlor than the male, in fact, the reverse seems indicated. However, due to the small number of animals, this cannot be statistically defined. It is interesting to note also, that a trend toward lower storage values during the exposure period seems indicated. Here again, and for the same reason, statistical significance does not obtain. Finally, it can be seen that upon cessation of exposure the loss of stored methoxychlor is complete within two weeks.

Discussion. From the data presented it would appear that the storage of methoxychlor in the fat of the rat occurs to a very limited extent and then only at relatively high doses. This is in sharp contrast to the storage of DDT. Thus a diet containing only 10 p.p.m. of this insecticide will cause the accumulation of 80 to 160 p.p.m. DDT depending on the sex of the animal(5). The finding of a low order of methoxychlor storage in fat is consonant with the low order of toxicity for the compound, and may be a partial explanation. However, in view of the fact that the oil solubility of methoxychlor is nearly identical with that of DDT, extensive storage in fat could be expected. It is therefore also possible that lack of storage of methoxychlor is a reflection of the rapid destruction(1) of the compound before storage can occur.

Summary. 1. Diets containing 100 and 500 p.p.m. methoxychlor reduce the growth rate and food consumption of young rats. 2. Storage of methoxychlor in the fat of the rat is minimal and can be demonstrated only at relatively high dietary levels.

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Antidiuretic Hormone in the Urine and Pituitary of the Kangaroo Rat.* (18216)

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The desert rodent *Dipodomys* ordinarily does not have access to water and its survival depends upon an extremely economical use of the water present in food or derived from the oxidation of hydrogen. Its ability to excrete a highly concentrated urine is the important factor in its conservation of water (1-5). This rodent can excrete a more concentrated urine than any other mammal whether terrestrial or marine. According to the Schmidt-Nielsens and their colleagues (2-5), the electrolyte-content of *Dipodomys* urine can reach 1.2 N and urea can be present in a concentration of 3.6 M. The mechanisms by which such unusual concentrations of electrolyte and urea are reached require elucidation. Howell and Gersh (1) concluded that considerable water was removed from the urine in both the collecting tubules and in the bladder. The most important hormonal factor in water conservation by the kidneys is the antidiuretic hormone of the posterior pituitary and it seemed probable that *Dipodomys* requires relatively large quantities of this hormone. A comparison of the urinary excretion of antidiuretic hormone and of the quantity of stored hormone in the pituitary of kangaroo rats *(Dipodomys merriami)* and of stock rats (Long-Evans strain) has been made and will be reported in this communication.

Methods. The kangaroo rats used in this study were obtained from the Arizona desert and were maintained on a diet of dry corn, barley, wheat and rolled oats. Lettuce was offered them 3 times a week but no drinking water was furnished. The animals maintained their weight which was taken to be good evidence of a satisfactory water balance. The comparative studies on stock rats were carried out with the Long-Evans strain of our laboratory colony. The problem of obtaining sufficient urine from the kangaroo rats which usually weighed 35 to 40 g was solved by ligating the neck of the bladder under ether anesthesia. Twenty-four hours later the operated animals were killed by illuminating gas and the accumulated urine was aspirated from the bladder by means of a syringe and fine needle. Approximately 0.5 ml of urine for assay could thus be obtained from each animal. Occasionally the urine was contaminated with blood although there was no apparent reason for hemorrhage into the urinary tract. The best specimens, never containing blood, were aspirated from the bladder at laparotomy before ligation; there was no means of ascertaining the period of collection of these samples. The samples of urine from ligated bladders represented the urine excreted during 24 hours. One-fourth per cent acetic acid was used for the preliminary dilution of the urine. Either the pars neuralis or the whole pituitary was removed under a dissecting microscope and extracted at room temperature by grinding with 0.25% acetic acid in an agate mortar. The diluted fine suspension was kept at 4°C before assay. (Brief extraction at 100°C immediately followed by chilling caused considerable loss of antidiuretic hormone.) Before extraction, the pars neuralis was quickly weighed on a sensitive torsion balance (± 0.02 mg); the whole pituitaries usually were not weighed.

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TABLE I. Concentration of Antidiuretic Hormone in the Urine of Kangaroo Rats and of Stock Rats (Long-Evans Strain).

No. of rats	Variety	Bladder ligated	Period of thirst (hr)	Mean and S.D. of antidiuretic hormone in urine as m.u./ml
9	Kangaroo	No		19.3 ± 13.3
11	,,	Yes		7.2 ± 4.0
3*	,,	,,		10.0
5	Stock	,,	0	<0.1
4	,,	,,	48	<0.2(3) 0.5(1)
44†	,,	No	48-72	1.7 ± 1.5†

* Blood in urine.

† 11 groups of 4 each.

Specimens from the stock rats were also collected in the same manner. In one experiment with 44 stock rats, urine was collected in metabolism cages each containing 4 rats. Antidiuretic hormone was determined in hydrated unanesthetized dogs by the method of Ames, Moore and van Dyke(6). The minimum quantity of hormone which could be detected was 0.25 m.u. (0.00025 U.S.P. unit).

Results. Antidiuretic activity was detected in all urines obtained from the kangaroo rats in concentrations ranging from 1.7 to 50 m.u. per ml (Table I). The average from 9 specimens of kangaroo rat urines before ligation of the bladder was 19.3 ± m.u. per ml. In the 24 hour urine collections from the ligated bladders of 11 rats the average titer was 7.2 ± 4.0 m.u. per ml. Three other specimens from ligated bladders contained gross blood and the average of these was 10.0 m.u. per ml. It was felt that small amounts of blood or serum in these 24-hour collections might account for their lower potency as compared with the urines obtained without ligation of the bladder.

Urine obtained from 5 stock rats on adequate food and water intake contained no antidiuretic activity. When drinking water was withdrawn for 48 hours or more, antidiuretic activity was detected in increasing amounts. There was a small amount of antidiuretic hormone (0.5 m.u. per ml) in only one of 4 rats deprived of water for 48 hours; the amount present in the urine of the other 3 was less than 0.2 m.u. per ml. Urine was collected for the last 24 hours of a 72-hour thirst period from 11 groups of 4 rats in metabolism cages. The average titer of these specimens, each representing 4 rats, was 1.7 ± 1.5 m.u. per ml and the range was 1.0 to 5.9 m.u. per ml. An acid pH was maintained in these urines by the use of collecting cylinders which contained 1% acetic acid. In a control experiment in which pitressin solutions of 2 and 5 m.u. per ml at pH 5.5 were allowed to drip through metabolism cages for a similar period, no loss of activity could be detected.

In Tables II and III, determinations of the amount of antidiuretic hormone in the neural lobe or whole pituitary are summarized. The dissection and weighing of the neural lobes was completed as rapidly as possible. Possibly there was some posterior lobe tissue not removed, especially in the small kangaroo-rat glands, which were found to contain more hormone in the whole gland

TABLE II. Antidiuretic Hormone in Neural Lobe of Kangaroo Rats and of Normal Stock Rats (Long-Evans Strain).

				Antidiuretic hormone in pars neuralis	
No. of rats	Variety	Avg body wt in g	Avg wt of pars neuralis in mg (fresh)	m.u./μg	m.u./gland ± S.D.
11	Kangaroo	41	0.37	.91	336 ± 110
14	Stock	217	1.07	.26	276 ± 146

TABLE III. Antidiuretic Hormone in Whole Pituitary of Kangaroo Rats and of Normal Stock Rats (Long-Evans Strain).

No. of rats	Variety	Period of thirst (hr)	Avg body wt in g	Mean and S.D. of total m.u. of antidiur. hormone in gland
29	Kangaroo		37	448 ± 128
9	Stock	0	249	275 ± 168
7	,,	48	232	320 ± 122
29	,,	72	205	490 ± 295

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6. Ames. R. G., Moore, D. and van Dyke, H. B., *Endocrinol.,* 1950, v46, 215.

,Table III). Anterior lobe tissue of the kangaroo rat, carefully freed of any neural lobe, contained no antidiuretic hormone. The fresh median eminence of a few rats of each species contained measurable quantities of antidiuretic hormone amounting to 19 m.u. in the kangaroo rat (average of 3 rats) and 11 m.u. in the stock rat (average of 7 rats). From a comparison of the neural lobes of the two species (Table II), it can be concluded that kangaroo rats have as much or more stored antidiuretic hormone than normal stock rats weighing 5 times as much. The observed difference is not statistically significant. The concentration of antidiuretic hormone is more than 3 times as high in the neural lobe of the kangaroo rat than in that of the stock rat.

It was concluded that for complete extraction of the pituitary antidiuretic hormone, whole pituitaries should be used. This plan was followed in the determinations reported in Table III in which an attempt was made to estimate the effect of thirst on the stored hormone in the stock rats. In comparison with normally-hydrated stock rats, kangaroo-rat pituitaries contain significantly more hormone ($P = < 0.01$ by Fisher's "t" test). Among the normal and thirsted stock rats, there was found a significantly increased storage of hormone after a thirst of 72 hours ($P = 0.05$).

A few other observations deserve mention. A number of inactivation experiments was completed. In every case, thioglycolate(6) destroyed the antidiuretic activity of urine; under the same conditions posterior-pituitary antidiuretic principle was likewise completely inactivated. Ultracentrifugation of 3 mixtures of kangaroo rat urine was also carried out. In each experiment there was marked sedimentation of the active substance. Before ultracentrifugation the average assay of these urine mixtures was 6.7 m.u. per ml. After ultracentrifugation the top fraction contained 1.2 and the bottom, 18.3 m.u. per ml.

Discussion. Since every urine specimen from the kangaroo rats had a detectable amount of antidiuretic activity it is evident that the secretion of this hormone may be one of the most important factors in the water economy of these desert rodents. It is probable that the kangaroo rat excretes highly concentrated urine in part because of a continuous and high rate of secretion of antidiuretic hormone by the pars neuralis. Our stock rats on an adequate fluid intake did not excrete antidiuretic hormone. However, after they were thirsted from 48 to 72 hours antidiuretic activity could be detected in the urine. The group of 44 rats which were thirsted for 72 hours appeared active and well at the end of this period despite some loss of weight. Increased secretion of antidiuretic hormone evidently induced adequate water conservation. However, if the concentration of antidiuretic hormone in the urine be taken as a measure of the rate of secretion, this rate of secretion is 4 times or more as rapid in the kangaroo rat on its normal diet than in stock rats of the Long-Evans strain thirsted for 48 to 72 hours. In another mammal, the dog, powerful osmotic stimulation is followed by a urinary titer of antidiuretic hormone not exceeding 6 m.u. per ml. The maximum concentration of hormone found in kangaroo-rat urine was 50 m.u. per ml in comparison with 6 m.u. per ml in thirsted stock rats. This high rate of secretion is associated with the storage of a large amount of hormone in the pars neuralis in a much higher concentration than found in the gland of stock rats. Gilman and Goodman(7) using rats for bio-assay injected either the standard posterior-pituitary solution or urine concentrates subcutaneously. They report an experiment in which the urine of laboratory rats thirsted 72 hours was thought to contain 100 m.u. of antidiuretic principle per ml. Urine concentrates contain substances which markedly retard absorption and prolong the action of any accompanying antidiuretic principle when injected subcutaneously. Hence, an assay such as that just cited is subject to gross error(8).

The work of Shannon(9) in the dog sug-

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7. Gilman, A. and Goodman, L., *J. Physiol.*, 1937, v90, 113.

8. Gilman, A., personal communication.

9. Shannon, J. A., *J. Exp. Med.*, 1942, v76, 387.

gests that supramaximal amounts of antidiuretic hormone are continuously secreted by kangaroo rats. This may depend upon a different renal response requiring larger amounts, a different rate of destruction of the hormone elsewhere, an excessive rate of secretion or a combination of more than one factor. The amount of antidiuretic hormone in the pituitary of stock rats was found to increase after a thirst of 72 hours. Thus, an increased rate of secretion of the hormone was associated with the storage of an increased amount of hormone in the gland. These results are difficult to compare with those of Simon(10) who found a decrease in pituitary oxytocic and pressor hormones in rats deprived of water for a much longer period (120-168 hours) than our rats (72 hours). He detected no significant change after 72-96 hours in a few rats; however, in view of the variations to be expected, this is not surprising.

Summary. The kangaroo rat *(Dipodomys merriami)* excretes large amounts of antidiuretic hormone in the urine (up to 50 milliunits per ml). The presence of this high concentration of hormone is believed to be related to the ability of this desert rodent to excrete the most concentrated urine of any mammal and to reflect a correspondingly high rate of secretion of antidiuretic hormone by the posterior pituitary. In 2 other mammals, the dog with powerful osmotic stimulation of the cerebrum, and the laboratory rat (Long-Evans strain) deprived of water for 48-72 hours, the maximum concentration of hormone in the urine is about 6 m.u. per ml. The hormone in kangaroo-rat urine undergoes sedimentation in the ultracentrifuge and thus resembles endogenous antidiuretic hormone in canine urine. The pituitary of kangaroo rats contains more antidiuretic hormone than that of normal laboratory rats although the latter are 5 to 6 times larger. After 72 hours of thirst the laboratory rat's pituitary contains an increased amount of hormone. Each microgram of fresh posterior lobe contains about 0.9 m.u. of antidiuretic hormone in kangaroo rats and about 0.3 m.u. in normal laboratory rats.

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10. Simon, A., *Am. J. Physiol.*, 1934, v107, 220.

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Effect of DDT Ingestion on Total Cholesterol Content of Ovaries Of White Rat.* (18217)

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Fitzhugh(1) observed that DDT, up to 600 p.p.m. in the diet of female rats, did not affect survival of young of the first generation. However, in the second generation of feeding DDT poisoned food, there were few living young at birth. Although the high mortality of young in Fitzhugh's DDT feeding tests may have come about through direct toxicity of the chlorinated hydrocarbon, it is possible that a hormonal disturbance may be involved in the whole problem. There is evidence that cholesterol is a precursor of some hormones exerting their influences in the reproductive cycle of the rat(2). Therefore, it seemed promising to investigate ovarian cholesterol in rats subjected to DDT poisoning. In connection with investigations (3) concerned with accumulation of ingested p,p′ DDT in various internal organs of white rats, additional females were exposed to diets

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* Journal Paper No. J-1816 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1102.

1. Fitzhugh, O. G., *Ind. and Eng. Chem.*, 1948, v40, 704.

2. Perlman, P. L., and Leonard, S. L., Proc. Soc. Exp. Biol. and Med., 1947, v66, 24.

3. Tauber, Oscar E., Hughes, Arden B., Tague, R. E., Pappas, Thomas R., and Warns, Thomas F., in preparation.

TABLE I. Accumulation of DDT and Content of Total Cholesterol in Ovaries of Rats Fed Diets Containing p,p′ DDT.

No. of rats*	Age (days)	DDT in diet (p.p.m.)	Duration of feeding DDT (days)	Organic chloride content, μg/g of tissue	Calculated DDT content, μg/g of tissue†	Total cholesterol, parts/1,000‡
6	90	—	—	594 (558-637)	—	—
3	90	—	—	—	—	6.7 (6.0-7.5)
4,3	90	100	60	—	364 (324-394)	3.9 (3.3-4.3)
3,3	90	300	60	—	1423 (1400-1444)	4.6 (3.7-6.0)
3,3	90	600	60	—	2594 (2570-2620)	4.1 (3.9-4.2)
3,3	41	800	11	—	4599 (4582-4676)	5.2 (5.0-5.3)
3,2	35	1,000	5	—	7492 (7304-7544)	6.2 (5.8-6.6)

* Where 2 figures are given, left hand number indicates sample size of rats for the DDT determinations, and right hand number is sample size of rats for total cholesterol analyses.

† These are "corrected" figures for DDT content, that is, the average normal organic chloride had been subtracted and these figures are the excess chloride presumed to have come from DDT present in the tissue at the time of analysis.

‡ Each of group averages is based on 6 determinations: 2 aliquots from extract of ovaries of each of the 3 rats.

of various levels of DDT. Ovaries of these rats were analysed for total cholesterol.

Materials and methods. Rats of Wistar-Ames stock were weaned at 25 days and placed on regular ration† and water for 5 days. At 30 days of age, female rats were placed on experimental diets, *ad libitum*, containing 100, 300, 600, 800, or 1,000 p.p.m. of pure p,p′ DDT (dichloro-dephenyltrichloroethane).‡ To insure even distribution of DDT throughout the feed, a carefully weighed amount of contaminant was dissolved in enough acetone to moisten 100 g of food, which, in turn, was then thoroughly distributed in 900 g of feed so that the resultant mixture would contain a known amount of DDT. Usually 1,000 g of each level of DDT were made as an experimental diet supply and kept in a covered container for 1 day. Before use for feeding, each supply was spread out on a large glass plate for at least a day, or until all odor of acetone had disappeared. Rats were kept on the 100, 300, and 600 p.p.m. diets for 60 days. Those on 800 and 1,000 p.p.m. had to be sacrificed after 11 and 5 days of exposure, respectively, when death was imminent. All rats were killed by overdose of ether. Ovaries were immediately removed and weighed. Tissues for DDT determinations were stored in vials of acetone and analysed by Carter's(4) method of measuring organic chloride. Tissues to be analysed for total cholesterol were transferred to vials containing a mixture of 3 parts ethyl alcohol to 1 part ethyl ether, in which they were stored at about 4°C until analysed by the dichromate-sulfuric acid oxidation method, essentially as described by Boyd(5) and Finkel(6).

Control rats were handled as described above except that their food contained no DDT. Ovaries from control rats were analysed for average normal organic chloride. This average was subtracted from the total organic chloride in ovaries from each experimental rat. The difference in chloride is presumably attributable to accumulation of DDT chloride. Average normal total cholesterol determinations were also made on ovaries from other control rats.

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† Ingredients, in %: ground yellow corn, 63.75; soy bean meal, 10.0; wheat middlings, 10.0; linseed oil meal, 8.0; dried skim milk, 5.0; alfalfa meal, 2.0; NaCl, 0.5; fine ground limestone, 0.5; vitamin A and D concentrate, 0.25. This diet was formulated by Dr. E. R. Becker, Iowa State College.

‡ Obtained from E. I. DuPont Co.

4. Carter, R. H., *J. Assn. Official Agri. Chem.*, 1947, v30, 456.

5. Boyd, E. M., *Am. J. Clin. Path.*, 1938, v8, 77.

6. Finkel, A. J., *Physiol. Zool.*, 1948, v21, 111.

Results and discussion. Pertinent data are tabulated. Figures in parentheses are ranges in each group of determinations. The average normal organic chloride (594 µg/g of ovary) was used as a "constant" in calculating the chloride presumed to come from DDT. Results for DDT content show a tremendous accumulation of toxicant in the ovaries. Especially surprising is the rapidity of storage at the 2 higher levels of diet, 800 and 1,000 p.p.m. even when duration of exposure was relatively brief: 11 and 5 days, respectively.

The average of 6.7 parts/1,000 of cholesterol (range 6.0-7.5) for control tissues compares favorably with Perlman and Leonard's (2) average of 7.3 parts/1,000 (range 5.0-9.0) based on ovaries from 10 non-pregnant rats. Although samples for each feeding level of the present investigation are small (3 rats, each) the average cholesterol value shows a decided decrease on the 100, 300, and 600 p.p.m. DDT diets. Because cholesterol is believed to have a part in the synthesis of reproductive hormones (probably progesterone, especially) this upset in the sterol picture may be of some consequence.

The deviations in cholesterol content at the 800 and 1,000 p.p.m. levels are likely of no significance, as such, and should not be compared with the results on the lower levels of DDT intake. Probably the short duration of exposure to DDT was not long enough to disrupt radically the sterol relationships in the tissues.

In the present series of experiments, the average weight of the 2 ovaries from 90-day rats was 66.8 mg; for 41-day rats, 44.4 mg; and for 35-day rats, 40.7 mg.

Summary and conclusions. Female albino rats, age 30 days at beginning of the experiments, were fed *ad libitum* ground feed diets containing 100, 300, or 600 p.p.m. of pure p,p′ DDT. After 60 days, at age 90 days, the rats were sacrificed, ovaries were removed, weighed, and analysed either for total cholesterol or for organic chloride. Control females were handled the same way except food was uncontaminated with DDT. Other groups of 30-day females were started on 800 and 1,000 p.p.m. DDT diets but had to be sacrificed after 11 and 5 days, respectively, when death was imminent. Normal average ovarian organic chloride from control rats was 594 µg/g of tissue (range 558-637); average ovarian total cholesterol was 6.7 parts/1,000 (range 6.0-7.5). Average weight of both ovaries from control 90-day rats was 66.8 mg. On 100, 300, and 600 p.p.m. diets, the average calculated DDT contents of the ovaries were 364, 1423, and 2594 µg/g of ovary; the total cholesterol values were 3.9, 4.6 and 4.1 parts/1,000, respectively. Rats on the diet of 800 p.p.m. for 11 days, and on the 1,000 p.p.m. for 5 days showed less change in total cholesterol values (5.2 and 6.2, respectively) than did the 90-day rats, exposed to the DDT diets for 60 days, perhaps because the shorter exposure to the toxicant was not long enough to disrupt the sterol metabolism of the ovaries. On the other hand, the DDT content of ovaries from rats on 800 p.p.m. averaged nearly 4600 µg/g of tissue; and on 1,000 p.p.m., nearly 7500 µg/g. These data indicate a "selective accumulation" of DDT by female gonadal tissue. The disturbance in the sterol balance of the ovary may be significant in view of the opinion that cholesterol may be a precursor of progesterone.

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Response of Tuberculin Skin Test to ACTH and Cortisone in Tuberculous Guinea Pigs. (18218)

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(Introduced by G. E. Burch)

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It has been shown that adrenocorticotrophic hormone (ACTH) and compound E (Cortisone) may modify tuberculin cutaneous reactions in guinea pigs sensitized with tuberculin(1) or heat-killed human tubercle bacilli(2), or infected with BCG tubercle organisms(3). These observations would seem to demonstrate the need for testing the effect of these hormones on the tuberculin reaction in the presence of active infection by virulent organisms.

Methods. Fifty-one guinea pigs (average weight 200 g) were infected by the subcutaneous injection into the groin of a suspension containing 0.1 mg virulent tubercle bacilli. The organisms had been recently cultured from patients with active pulmonary tuberculosis. All animals subsequently showed definite evidence of the presence of tuberculous infection, manifested by localized or generalized lymphadenopathy (1+), buboes (2+), draining sinuses (3+), or ulceration in the groin (4+) (Table I); in all animals tested acid-fast organisms were found on smear of exudate discharged from lesions, and in all cases additional confirmation of tuberculous infection was discovered at necropsy (results to be reported separately). The animals were divided into 3 groups of 17 each. The animals in Group 1 received daily subcutaneous injections of 1 mg of Cortisone; injections were begun one day before infection by tubercle organisms and continued until the observations were completed. Animals in Group 2 were used to observe the effects of varying dosages of ACTH and Cortisone on the tuberculin reaction. The animals in Group 3 served as controls. Selected animals of the control group were challenged at intervals with intradermal injections of 0.005 mg of Purified Protein Derivative (0.1 cc of the 2nd strength) and 6 weeks after infection all animals in this group showed a strongly positive cutaneous reaction. Then the animals of Group 1 received the challenging intradermal test, and the animals of the second group were utilized in short term experiments. The tuberculin reactions were read 24, 48 and 72 hours after introduction of the test dose and classified as 1+ (erythema and induration), 2+ (erythema, induration and hemorrhage), and 3+ (erythema, induration, hemorrhage and necrosis). In representative cases photographs were made and the lesions excised for histopathologic study.

TABLE I. Effect of Cortisone on Tuberculin Reaction. 17 animals in each series. (1 mg daily from beginning of infection).

	Degree of infection*				Tuberculin reaction		
Group	1+	2+	3+	4+	1+	2+	3+
			Cortisone				
1	2	4	10	1	6	8	3
			Controls				
3	1	6	3	7	4	9	4

* See text for explanation of symbols indicating degree of reaction.

Results. The animals in Group 1 (which received 1 mg Cortisone daily) manifested tuberculin reactions not significantly different grossly and microscopically from those in the controls.

The histologic picture (Fig. 1) consisted in a severe inflammatory reaction in the epidermis, dermis and underlying muscle tissue. There was dilatation of small blood vessels with swelling of their endothelium, together with a large outpouring of exudate. This exudate was composed principally of neutrophiles,

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1. Favour, C. B., quoted by Forsham, P. H., First Clinical ACTH Conference. Philadelphia, The Blakiston Company, 1950, pp. 520-521.

2. Stoerk, H. C., *Fed. Proc.*, 1950, v9, 345.

3. Harris, S., and Harris, T. N., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 186.

FIG. 1.
Reaction to tuberculin skin test after 6 weeks of cortisone (1 mg/day).

lymphocytes and macrophages, with only an occasional eosinophile being present. The exudate was diffusely disseminated but there was a notable tendency for perivascular and perineural accumulation. The subcutaneous connective tissue was edematous. Many of the collagen bundles in the dermis were fragmented and stained poorly. There was ulceration of the epidermis immediately overlying the more intense inflammatory zones and in these areas considerable necrosis was noted with almost complete destruction of the cutaneous appendages.

In Group 2, Cortisone was administered intramuscularly in divided doses twice daily to 3 pairs of animals. To 1 pair, 10 mg daily per animal was given for 3 days; the animals were tested on the 2nd day; both had strongly positive cutaneous reactions on the 4th day, identical in intensity (2+ and 3+) with results of control tests made in the same animals prior to administration of the hormone. A 2nd pair received 10 mg daily per animal for 4 days; the animals were tested on the 3rd day; both had strongly positive reactions (2+ and 3+) on the 5th day, the same result as prior to administration of the drug. The 3rd pair received 20 mg daily per animal for 3 days; the animals were tested on the 2nd day; both showed weakly positive cutaneous reactions (1+). In all animals the sections for biopsy were identical with those from the controls, with the exception of the last pair, 1 of which showed less inflammatory reaction (Fig. 2).

Two animals received 5 mg ACTH daily for 4 days, in the same manner as for Cortisone and were tested on the 2nd day. Both had strongly positive reactions (2+) on the 4th day, and sections for biopsy were not different from those of the controls. Five animals, having first been given control skin tests, received 10 mg ACTH daily (2.5 mg/6 hours) for 8½ days; the animals were tested on the 7th day, with the following results (Table II): in 1 animal the previously strong-

FIG. 2.
Reaction to tuberculin skin test after 3 days of cortisone (20 mg/day).

TABLE II. Effect of ACTH on Tuberculin Reaction. (10 mg daily for 8½ days).

	Prior to ACTH		After ACTH	
Animal	24 hr	48-72 hr	24 hr	48-72 hr
1	2+*	3+	3+	3+
2	3+	3+	1+	1+
3	1+	2+	1+	1+
4	1+	2+	1+	1+
5	3+	3+	(Died 40 hr after test)	

* See text for explanation of symbols indicating degree of reaction.

ly positive reaction was unchanged, 3 animals showed weakly positive reactions with intensity definitely less than prior to ACTH; and the 5th animal died 40 hours after administration of the challenging test with a negative reaction. Results of histologic studies of the specimens for biopsy were in accord with the gross observations, *i.e.*, sections from the first animal showed severe inflammatory reaction, from animals 2, 3 and 4 minimal inflammatory reaction, and from the 5th animal a normal appearance (Fig. 3). In those animals in which there was microscopic evidence of a less severe reaction the principal change was one of degree; if only a minimal inflammatory reaction was present, the exudate was composed principally of lymphocytes and macrophages with few neutrophiles.

Discussion. With respect to dosage, Hench and associates(4) suggested that 75 to 100 mg of Cortisone may represent the minimum effective daily dose in adult human beings (equivalent to 1.0 to 1.4 mg/kg); since our animals averaged 1/5 kg in weight, a dose of 1 mg a day (equivalent to 5 mg/kg) would represent a 4 fold increase over the minimum human dose, provided the dosage could be transferred to that species. A difference in species' response is indicated by the protection of rats against the "anaphylactoid reaction" to egg albumin by both ACTH and Cortisone(5), and the failure to protect guinea pigs against anaphylactic shock by ACTH* (6). Of the various dosages tried the only ones effective in modifying the tuberculin reaction were 10 mg ACTH daily (2.5 mg intramuscularly every 6 hours) for 7 days which modified the reaction in 4 of 5 animals and (possibly) 20 mg Cortisone (10 mg intramuscularly twice daily) for 3 days which apparently modified the response in 1 of 2 animals.

Numerous investigators(8) have shown that there is no correlation between the in-

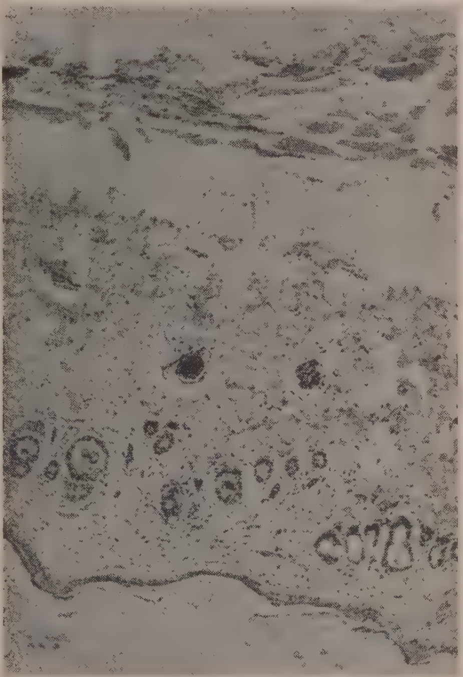

FIG. 3.
Negative reaction to tuberculin skin test.

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5. Selye, H., *Canad. M. A. J.,* 1949, v61, 553.

* At the time these experiments were undertaken we could find no information on the dose of the hormones in the guinea pig; and, although later studies by us and by others(7) have shown much larger doses are necessary, 1 mg Cortisone daily seemed to be a reasonable starting dose).

6. Leger, J., Leith, W., and Rose, B., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 465.

7. Thorn, G. W., Forsham, P. H., Frawley, T. F., Hill, S. R., Jr., Roche, M., Straehelin, D., and Wilson, D. L., *New England J. Med.,* 1950, v242, 783, 824, 865.

8. Boquet, A., and Bretey, J., *Ann. Inst. Pasteur,* 1934, v52, 252.

tensity of the tuberculin reactions and the severity of the tuberculous infections, and that the tuberculin reaction appears after a variable latent period, rises sharply in intensity, levels off, and then diminishes, perhaps disappearing at the time of death of the animal; therefore, we are unable to attribute the absence of the reaction in the one animal (which died 40 hours after the test) to the administration of ACTH.

Summary. (1) ACTH and Cortisone when given in proportionately large doses modified but did not abolish cutaneous tuberculin reaction in guinea pigs infected with virulent tubercle organisms. (2) Following administration of ACTH and Cortisone the histopathologic changes consisted in a quantitative diminution in the inflammatory exudate rather than a qualitative change in its cytologic structure.

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Development of Embryonic Guinea Pig Mullerian Ducts in Anterior Eye Chamber Grafts.* (18219)

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The problem of the relation of sex hormones to sexual differentiation in mammals has received the attention of many investigators who have utilized fundamentally different types of approach in their studies. The injection of sex hormones into the pregnant female(1-5), the treatment of developing pouch young in marsupials(6-9), gonadectomy during development(10-13), and the transplantation of embryonic tissues to postnatal hosts in different sexual conditions(14, 15) have all contributed new information on the subject. However, unanimity of opinion regarding the necessity for sex hormone action for proper sex differentiation does not yet exist. In 1945 I began a study of the relation of sex hormones to uterine gland development in the guinea pig by comparing the conditions found in normal newborn and later stages with those subjected to ovariectomy at birth, and with others treated by estrogen injections(16). Although the uterus of the newborn guinea pig readily responded to treatment with estrogens, it became clear that critical stages occurred earlier in development since uterine glands were already present in the guinea pig at birth.

In order to examine the possibilities for the differentiation of the uterus, and especially of uterine glands, in the absence of all hormones secreted by sex glands, portions of the embryonic reproductive duct system were removed from embryos of timed development

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TABLE I. Data of Results from 23 Anterior Chamber Grafts.

Series	Embryo age	Graft duration, days	Age equiv., days	Donor embryo	Host	Observations		
						M.D.	Ut. gl.†	W.D.
2A	50 d.	30	12	♀	♂ *	×	?	
					♀	×	++	
					♂	×	++	
2B	50 d.	30	12	♀	♀ *	×	+++	
					♀	×	+++	
					♂	×	+++	
3A	50 d.	25	7	♀	♀ *	×	++	
					♀ ‡	×	×	
					♂	×	0	
3B	50 d.	25	7	♀	♀ *	×	++	
					♂ *	×	0	
					♀ ‡	×	×	
					♂	×	0	
5A	38 d.	29	0	♀	♀ *	×	+	
	8 hr				♂ *	×	0	
4A	33 d.	35	0	♂	♀ *	×	0	
	10 hr							
4B	33 d.	35	0	♂	♀	×	0	×
	10 hr							
6A	31 d.	37	0	♀	♀ *	×	+	
	4 hr				♂ *	×	0	
					♂	×	+	
6B	31 d.	37	0	♂	♀ *	×	++	
	4 hr				♂ *	×	0	×
					♂	×	0	

* Castrated or spayed.

† Symbol: + development equal to 0 day normal ♀.

‡ Female became pregnant. Evaluation of gland development impossible because of overgrowth of uterine submucosa.

and transplanted into adult hosts in different hormonal conditions. Using a modification of the technic employed by Greene(17), transplants of portions of sex ducts from males or females were placed in the anterior chamber of the eye of previously castrated male and spayed female hosts. For comparison transplants were also made in the anterior chamber of both normal males and females.

Methods and materials. Tissues for transplantation were obtained from embryos removed from the uterus of a pregnant female guinea pig. Embryos were dissected in cold sterile saline within an hour after their removal, and tissues transferred to the anterior chamber of a host guinea pig through a corneal slit external to the iris. Castrated hosts had been operated at least 30 days previously.

Sixty-six grafts have been recovered from 94 transplants. Of these 29 have been studied. Tissues used for transplants have been removed from male or female embryos of 25-days to 50-days postcopulation age. Tissues studied have been obtained from embryos of 31-days to 50-days, and recovered as grafts from the anterior chamber 25 days to 37 days later. According to Bookhout(18), the gonad in the guinea pig is indifferent in structure at 22 days of embryonic life; the testis becomes recognisable about the 25th day. I have been able to distinguish the sexes of dissected embryos by day 38. When tissues for transplantation were removed from embryos of earlier ages, it was necessary to transplant the embryonic gonad to a separate host for determination of the sex of the embryo from which the transplant originated. The age of the recovered graft tissue is in terms of the equivalent expected date of birth on the basis of a 68-day gestation period. Thus, transplants from a 31-day embryo, persisting as a graft for 37 days are rated

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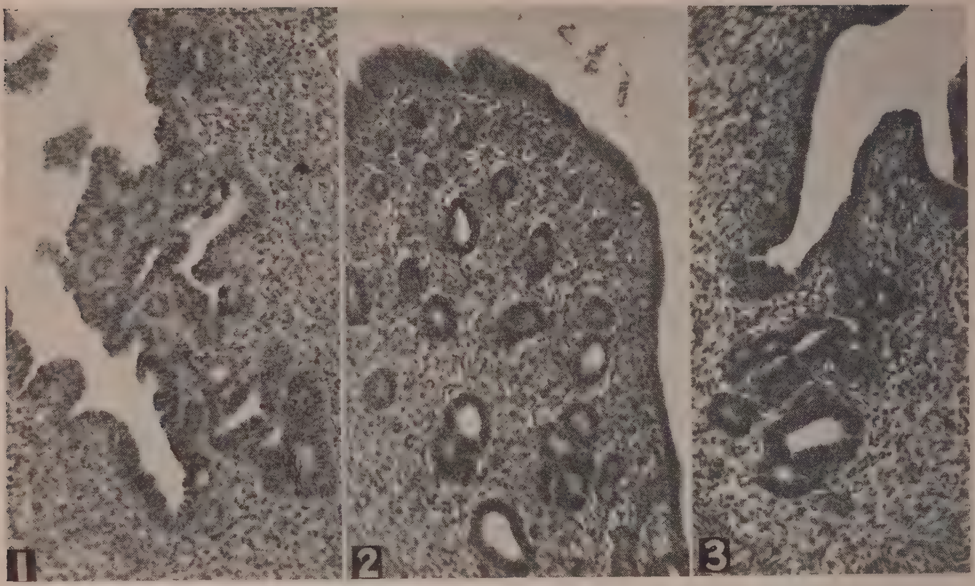

Sections through Mullerian duct graft tissue showing glandular development.
FIG. 1. Female tissue (31-day embryo) existing as graft in spayed female host for 37 days.
FIG. 2. Female tissue (50-day embryo) existing as graft in normal male host for 30 days.
FIG. 3. Male tissue (31-day embryo) existing as graft in spayed female host for 37 days.

as the equivalent of normal tissues at birth, or zero days; while transplants from a 50-day embryo, persisting as a graft for 30 days, are rated as equivalent to that of a 12-day postnatal guinea pig.

Results. Data covering 23 grafts of reproductive ducts studied thus far are listed in Table I. The study of normal embryos reveals that the Mullerian duct has not formed in a 24-day guinea pig. Both ducts are present and well developed in embryos of both sexes of ages 31 days and 33 days. By 37 days, in a normal female, the Wolffian duct has degenerated to a mere outline of tissue. In 60-day female embryos the uterus is not completely formed, but gland development is beginning at the extreme anterior portion.

Mullerian duct persistence. Sex duct tissues obtained from female embryos of 31 days to 50 days of age and transplanted to different hosts were removed as grafts from the anterior chamber of the eye at ages equivalent to birth or 12 days postnatal. Eighteen grafts contained recognisable uterine tissue and among these, 12 grafts had definitely formed uterine glands. These grafts were removed from normal males, normal females and spayed female hosts. Fig. 1 shows a section of uterine tissue of a 31-day female embryo recovered as a graft from a spayed female host 37 days later. Fig. 2 shows a section of uterine tissue of a 50-day female embryo that was recovered as a graft from a normal male host 30 days later. Thus far definite uterine glands have not been identified in grafts removed from castrated male hosts. Sex duct tissues obtained from male embryos yielded 5 grafts in which uterine tissue was recognisable and among these, one graft contained uterine glands. This graft had persisted in the eye of a spayed female host for 37 days (age equivalent—birth) after its transplantation from a male embryo 31 days of age. A section of this graft is illustrated in Fig. 3.

Wolffian duct persistence. Developed Wolffian duct tissue was present in the 4 youngest embryos only, ages 31 days and 33 days. The 3 grafts from the female contained no

Wolffian tissue. In the 5 grafts of transplanted embryonic male tissue, only 2 grafts contained recognisable Wolffian duct. One of these grafts was recovered from a normal female host; the other, from a castrate male host. In the latter graft a small piece of testis was present that had been inadvertently carried over with the original transplant.

Discussion. The results show that the Mullerian duct of 31-day to 50-day guinea pig donors transplanted to the anterior chamber will persist and continue to differentiate. Mullerian duct tissues from female donors differentiated into a recognisable uterus in all types of hosts. The differentiation of the uterus seems to be independent of the influence of host hormones since uterine glands, equal in development to that of normal females, were found in grafts from normal males, normal females and spayed females. Of even greater interest is the fact that tissue from a 31-day male donor in a spayed female host differentiated into a uterus with well developed glands. The results indicate that ovarian hormones are not necessary for the development and differentiation of the uterus, and that testicular hormone does not inhibit it.

Summary. (a) Anterior chamber grafts of embryonic guinea pig female reproductive ducts developed into uterus in all types of hosts. Definite uterine glands were found in grafts from all hosts except the castrate male. (b) Similar grafts of embryonic male reproductive ducts developed as Mullerian tissue, and in one case definite glands were present; in only 2 of these grafts was Wolffian tissue present also.

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Enhancement of Spreading Cortical Depression by a Dialyzing Technic
(18220)

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Leão first described a response to stimulation of the exposed cerebral cortex of rabbits which he called spreading cortical depression (1). This phenomenon follows electrical or mechanical stimulation and manifests itself as a slowly migrating temporary reduction in the normal electrical activity of the cerebral cortex. Another manifestation of the depression wave is an associated transient reduction (7-15 Mv.) in the normal "D. C" potential of the cerebral cortex(2).

Although the rabbit is peculiarly susceptible to this reaction, protecting the cerebral cortex of cats and monkeys from exposure to room air by means of mineral oil effectively opposes the appearance of this response(3). However, it has been demonstrated in this laboratory that cooling the mineral oil covering the cerebral cortex makes it possible to elicit spreading depression with a high degree of regularity in rabbits, cats, and monkeys (4). In addition, the sequential phases of the migratory depression have been characterized and identified on the basis of the associated reduction in cortical "D. C." potential (4). The same designations have been used in identifying the "D. C." voltage changes (see Fig. 1) obtained in the experiments reported herein. The first important negative "D. C." potential change occurs at the proximal electrode E_1 (solid line) situated on the

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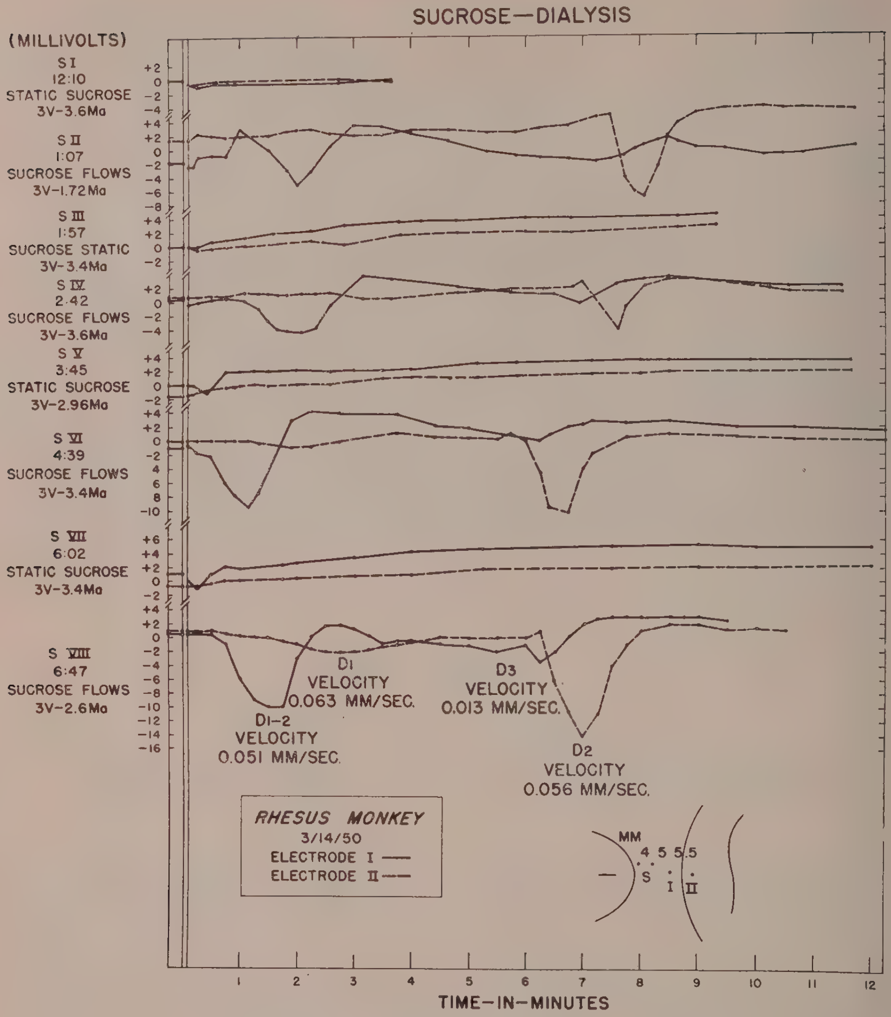

FIG. 1.

Illustration of spreading depression as indicated by the fall (in millivolts) of the cortical D.C. potential. Those stimuli labeled sucrose flows, illustrate propagated depressions following dialysis. Those stimuli labeled static sucrose, show that no depression occurred when dialysis did not precede stimulation.

precentral convolution. This reaction is labeled D_{1-2}. The next large "D. C" millivolt depression is recorded on the distal pick up electrode E_2 (dotted line) located on the postcentral gyrus. This reaction is labeled D_2 and indicates that the spreading depression has been propagated from the precentral to the postcentral convolution. The D_1, D_2, D_3, and D_4 reactions obtained in these experiments were also seen in the cooled mineral oil experiments and are mentioned to point out that similarity.

The experiments here described will demonstrate that the production of these same phenomena is aided by a virtual dialyzing procedure. The dialysis-like technic depended on the flow of a 9.95% sucrose solution across the surface of the arachnoid membrane.

TABLE I. Summary Table of Percentages.

Dialysis	No. of stimuli	Depressions on both electrodes $D_{1\text{-}2}$ and D_2 reactions are separated, %	 $D_{1\text{-}2}$ and D_2 reactions are simultaneous, %	 Total %	$D_{1\text{-}2}$ reaction on E_1 only, %	Depressions unrelated to stimuli, %	No depression obtained on either electrode, %
With	48	64.5	12.5	77	20.8	2.0	.0
Without	36	22.2	2.7	24.9	22.2	5.5	47.2

Method. Eight experiments were performed on 4 monkeys (*Macaca mulatta*) anesthetized with intraperitoneal dial (0.45 cc/kilo). Amplification and recording of the electrocorticogram as well as cortical "D. C." potential variations involved the same instrumentation described previously(3). The technic employed in these experiments depended on the construction of a plaster of Paris wall on the bone around the exposed brain. The wall contained tubes which permitted the continuous exchange of a 9.95% solution of sucrose in distilled water (isosmotic with human blood serum). After 20 to 30 minutes of exposure to the sucrose an electrical stimulation was given and the results recorded. The sucrose was then replaced by 2 to 4 changes of a phosphate buffered Ringer-Locke solution. After 20 to 30 minutes this physiological solution was replaced by 9.95% sucrose and another electrical stimulation was given immediately. In the last 2 experiments the phosphate-buffered Ringer-Locke solution flowed continuously over the cortex prior to its replacement by sucrose.

The technic thus consisted in giving one stimulus after a dialyzing procedure (flowing 9.95% sucrose) and then exposing the arachnoid surface to a physiological salt solution prior to another stimulus administered at once in static 9.95% sucrose without dialysis.

Results. Fig. 1 is a graphical representation of the "D. C." millivolt changes following electrical stimulation. Those stimuli labeled "static sucrose" (done immediately after replacing the salt solution with sucrose) produced no "D. C." millivolt reduction or spreading depression. In contrast, the stimuli designated as "sucrose flows" (done after 20 to 30 minutes of exposure of cortex to flowing sucrose) produced the typical propagated depression of electrical activity and "D. C." voltage.

The summary table of percentages indicates that all of the 48 stimuli given after flowing sucrose did not produce the ideal propagated depression response. Thus, 12.5% of the stimuli caused reactions which occurred on both electrodes simultaneously (or nearly so), and 20.8% gave an isolated reaction on the proximal electrode only. However, whereas 77% of stimuli following the dialytic method produced depressions on both electrodes, only 24.9% of those stimuli without dialysis produced reactions on both electrodes. In addition, 47.2%, or nearly half, of the stimuli which did not have benefit of sucrose exchange produced no variety of depression. In contrast to this, nearly 100% of all the stimuli following sucrose dialysis produced some variety of depression.

It is interesting that the $D_{1\text{-}2}$ reaction on E, occurred following 20-22% of stimuli without regard to the use of dialysis. However, the average amplitude of this reaction after dialysis was 10.6 Mv. as compared to 5.3 Mv. in the control series. Therefore, the dialytic method did not increase the incidence of the $D_{1\text{-}2}$ reaction on E, but did increase its amplitude by 100%. It is significant that the average 100% increase in amplitude of the $D_{1\text{-}2}$ reaction did not suffice to make it propagate across the central sulcus to E_2.

A comparison of the amplitudes of propagated depressions with and without dialysis revealed another significant fact. The average total millivolt depression for the $D1_{-2}$ reaction following dialysis was 11.3 Mv. and propagation occurred in 64.5% of 48 stimuli. Without dialysis the same reaction averaged 5.3 Mv. and successfully migrated to E_2 in 22.2% of 36 stimuli. The D_2 depression when present on E_2 had an average amplitude of 9.0 Mv. with and 6.7 Mv. without sucrose exchange. Here, then, is evidence that spreading depression is not an all or none phenomenon. It can occur at the electrode nearest to the stimulators without propagating to the postcentral gyrus and when propagation does occur the amplitude of the reaction can vary in terms of the "D. C." millivolt swing.

There is some indication in our records that the brain wave amplitude decreases after exposure to static sucrose and increases somewhat when the sugar is continuously flowed through the pool. This effect could well be explained by electrolyte entering the stationary sugar medium from cerebrospinal fluid or brain and creating a partial shunt resulting in an apparent decrease in brain wave amplitude.

Therefore, substitution of Ringer-Locke solution for sucrose results in almost complete shunting of the electrocorticogram, creating a nearly isoelectric recording. In many experiments the above procedure caused discrete spikes to appear, especially on E_1, so that an otherwise isoelectric recording was punctuated at varying intervals by low amplitude oscillations.

In the last 2 experiments, the Ringer-Locke solution was permitted to flow continuously instead of remaining static during the period prior to control stimuli. Whereas 40% of the stimuli given after static Ringer-Locke exposure produced no response, 63.6% of the stimuli after flowing Ringer-Locke treatment yielded no type of depression. This result indicates that sucrose is not removing a non-electrolyte extractive but rather is attracting one or more of the ions present in the subarachnoid space and replaceable by the Ringer-Locke solution. This interpretation seems tenable, since exposure to the buffered physiological salt diminishes the incidence of spreading depressions and increases the number placed in the "no type of depression" category.

Discussion. The mechanism by which the sugar solution increases the incidence of propagated depressions remains to be conclusively demonstrated. Exposure to the physiologically balanced ion solution following the dialytic method appeared to decrease the incidence of spreading depression to a significant extent. This result suggests that the removal of one or more of the ions present in the subarachnoid space creates a deficient chemical environment conducive to an increased incidence of the spreading depression phenomenon. The postulated deficiency could be due to a simple reduction in electrolyte concentration or the inhibition of an enzyme system by the loss of an ion activator.

Summary. Exposure of the rhesus monkey's cerebral cortex to a flowing 9.95% sucrose solution significantly increases the incidence of the spreading depression reaction evoked by electrical stimulation.

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Relationship of Coagulase-Globulin to Prothrombin. (18221)

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In previous reports(1,2) we have shown that *staphylocoagulase* reacts with a plasma globulin factor, *coagulase-globulin,* to form a thrombin-like substance, *coagulase-thrombin. Coagulase-globulin* is, by itself, unable to clot pure fibrinogen. The reaction of *staphylocoagulase* with *coagulase-globulin* to form the active clotting agent yields hyperbolic time-concentration and conversion curves, similar to those obtainable for the conversion of prothrombin to thrombin. Because of other similarities, as well as some differences, in their action, it seemed necessary to investigate what part, if any, pro-

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thrombin plays in the staphylocoagulase reaction, and to determine the relationship of *coagulase-globulin* to prothrombin and to prothrombin activity.

Experimental. I. *Methods.* (a) *Coagulase-globulin (CG) titer*, expressed as the reciprocal of the highest dilution of oxalated plasma giving 4+ coagulation of a system composed of 0.5 cc of tested material in serial dilution in imidazole buffered saline pH 7.4 + 0.5 cc of 2% fibrinogen (Fraction I-2A) + 0.1 cc of standard staphylocoagulase(1). It should be noted that only fibrinogen free of CG may be used in this titration. (b) Prothrombin activity, according to the method of Warner, Brinkhous, and Smith(3) as modified by Hurn and Mann(4). (c) All determinations carried out at 35°C. (d) All plasma centrifugations in the cold at 3000 r.p.m. for 30 minutes.

II. *Materials.* (a) *Fresh plasma:* from 4.5 cc of human blood plus 0.5 cc 0.1 M sodium oxalate, by centrifugation at 3000 r.p.m./30 minutes. (b) *CaF_2 plasma*: 0.3 cc of M/CaF_2 suspension is centrifuged strongly and the supernatant fluid completely drained away. The sediment is mixed with 1 cc of plasma, incubated with intermittent stirring at 37°C for 15 minutes, then centrifuged. Occasionally it is necessary to repeat this procedure twice in order to remove the last traces of prothrombin. (c) *Defibrinated plasma*: by the addition of thrombin (Upjohn), 15 units/cc of plasma. (d) *Defibrinated CaF_2 plasma*: plasma from (c) treated with CaF_2 as in (b). (e) *$Ca_3(Po_4)_2$ plasma*: 1 cc of fresh plasma added to centrifuged sediment from 1 cc of freshly prepared and thoroughly washed 0.008 M suspension of $Ca_3(Po_4)_2$, incubated for 10 minutes at 37°C, then separated by centrifugation. (f) *$Al(OH)_3$ plasma*: fresh plasma to which is added an equivalent of 1% freshly prepared and thoroughly washed $Al(OH)_3$, incubated at 37°C for 15 minutes, then separated by centrifugation. (g) *$BaSO_4$ plasma*: 100 mg of $BaSO_4$/cc of plasma, stirred for 15 minutes at 37°C, then separated by centrifugation. (h) *Seitz plasma*: fresh plasma filtered through Seitz filter (EK Germicidal Pads). Five filtrations of 100 cc of plasma through at least 3 pads usually adequate to remove all prothrombin.

TABLE I. Effect of Adsorption on Coagulase-Globulin Titer and Prothrombin of Oxalated Human Plasma.

Plasma	CG titer	Prothrombin units
Fresh (control) (a)	10^4	330
CaF_2 (b)	10^4	0
Defibrinated (c)	10^4	343
Defibrinated CaF_2 (d)	10^4	0
$Ca_3(Po_4)_2$ (e)	0	0
$Al(OH)_3$ (f)	0	0
$BaSO_4$ (g)	0	0
Seitz (h)	10^5	0

Results. As shown in Table I, human oxalated plasma can be freed of prothrombin by adsorption with CaF_2, but this treatment does not affect the coagulase-globulin. On the other hand, the other adsorbants, $Ca_3(Po_4)_2$, $Al(OH)_3$, and $BaSO_4$, are active adsorbants of both coagulase-globulin and prothrombin.

A second dissociation of prothrombin and coagulase-globulin can be demonstrated by filtration of plasma through a Seitz filter. Successive filtrations reduce the prothrombin content to variable degrees(5), but do not affect the coagulase-globulin titer.

Discussion. A dissociation between coagulase-globulin and prothrombin can be demonstrated by means of adsorption on CaF_2 as well as by repeated filtrations through a Seitz filter. This, plus the previously described(2) differences between the activity of prothrombin and of the staphylocoagulase system in the presence of substances having anti-prothrombin and anti-thrombin activity (Chlorazol fast pink, oxalates, citrates, soluble fluorides, and heparin) indicates that the conversion of *coagulase-globulin* to the clotting agent *coagulase-thrombin* is independent of prothrombin and of prothrombin activity, and that coagulase-globulin is not iden-

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tical with prothrombin.

Summary. A further dissociation between coagulase-globulin and prothrombin has been demonstrated by means of adsorption on CaF_2 and by repeated filtrations through a Seitz filter. This indicates that coagulase-globulin is not identical with prothrombin.

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Ovarian and Lymphoid Tumors in Female Mice Subsequent to Roentgen-Ray Irradiation and Hormone Treatment.[*] (18222)

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Ovarian tumors of the granulosa cell type have developed in mice subjected to total body irradiation(1) with roentgen rays and after intrasplenic transplantation of ovaries into castrated mice(2). The development of ovarian tumors under the latter experimental conditions has led to the hypothesis that prolonged exposure to excessive amounts of gonadotrophic hormone of the pituitary constitutes a carcinogenic stimulus(3). Ovarian tumors did not appear when estradiol benzoate or testosterone propionate were injected into mice with intrasplenic ovarian grafts. Furthermore, preliminary experiments indicated that gonadal hormones prevented the appearance of ovarian tumors in mice subjected to roentgen irradiation(4).

Many investigators, since the original observations of Furth(5), have noted the leukemagenic action of x-rays in mice and more recently the effects of additional treatment with estrogens(6).

Materials and methods. Three groups of 50 female mice of the BC[†] stock were used in the following experiments. The groups were so constituted that littermates were distributed throughout all groups as uniformly as possible. At ages ranging from 34 to 92 days the mice received a total body irradiation of 280 to 380 r. An equal number of mice in each of the groups received comparable doses. From 42 to 45 animals in each group survived irradiation, the earlier periods of the experiment and are included in the following data. The animals were maintained in an air conditioned room (72-74° F temperature and 50-60% relative humidity). They were fed a commercial food (Purina Fox Chow) and water *ad libitum.* The mice were examined daily throughout the course of the experiments. One group received weekly subcutaneous injections of 0.05 cc of sesame oil, one group 1.25 mg of testosterone propionate[‡] dissolved in 0.05 cc of sesame oil; and one group 16.6 μg of estradiol benzoate[§] dissolved in 0.05 cc of sesame oil. Injections were omitted on rare occasions in

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[*] This investigation has been supported by grants from The Anna Fuller Fund, the Jane Coffin Childs Memorial Fund for Medical Research and the United States Public Health Service.

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[†] BC refers to mice obtained as follows (PM♀ x C_3H♂) (F1♀ x PM♂) and subsequent brother to sister inbreeding. "PM" refers to mice sent to the writer by Dr. H. B. Andervont and derived from the colony of Drs. Pybus and Miller in England. The mice used in the present study were from the eleventh to the fifteenth generations of inbreeding—and largely from the twelfth to the fourteenth.

[‡] The testosterone propionate was obtained from Organon, Inc., through the courtesy of Dr. Kenneth Thompson.

[§] The estradiol benzonate was obtained from Ciba Pharmaceutical Products, through the courtesy of Dr. E. Oppenheimer.

TABLE I. Mice with Ovarian Tumors and Average Survival Time of Mice of the BC Strain Subjected to Total Body Irradiation and Treated with Hormones.

	Avg at death			
Group	Without tumors	With tumors	Small tumors†	Large tumors
Sesame oil	320(21)*	513(24)	496(9)	524(15)
Testosterone propionate	381(16)	512(29)	506(9)	515(20)
Estradiol benzoate	295(42)	—	—	—

* Numbers in () indicate the No. of mice.
† Tumors measuring <5 mm greatest diameter.

TABLE II. Survival and Number of Ovarian and Lymphoid Tumors in Irradiated Mice (280-380 r) Given Weekly Subcutaneous Injection of 16.6 μg of Estradiol Benzoate or 1.25 mg of Testosterone Propionate Dissolved in 0.05 cc of Sesame Oil or Sesame Oil Alone.

Age group	Estradiol benzoate	Testosterone propionate	Sesame oil
100-199	7(–/4)*	1(–/1)	2(–/1)
200-299	18(–/11)	4(–/2)	9(–/8)
300-399	8(–/6)	4(1/3)	7(2/6)
400-499	9(–/2)	15(10/3)	8(6/3)
500-599		20(17/–)	18(15/3)
600-699		1(1/–)	1(1/–)
Total	42(0/23)	45(29/9)	45(24/21)

* Figures in () indicate the number of ovarian tumors/No. of lymphomas.

the latter group when the general condition of an animal seemed to contraindicate treatment for one or 2 weeks. At necropsy the ovaries of all animals were fixed in Bouin's fluid and after sectioning they were stained in hematoxylin and triosin. Other tumors and organs were saved and similarly prepared when the gross observations indicated some abnormality.

Observations. More than one-half of the irradiated mice given weekly injections of testosterone propionate dissolved in sesame oil, or sesame oil alone, had ovarian tumors at death; and none of the estrogen-treated mice had ovarian tumors (Table I). Although the average ages of the mice of the estrogen-treated group was much less than that of the other two groups 17 mice of the former groups survived as long as did 19 of the mice with ovarian tumors in the 2 latter groups (Table II). The early death of mice of the estrogen-treated group was due primarily to the early appearance and high incidence of leukemia. Some of the small ovarian tumors were so small that they were diagnosed only after examination of the serial sections of the ovaries. They were composed of from 1 to 3 nodules of granulosa cells, usually less than 1 mm in diameter. Histologically they were not unlike the larger tumors.

All of the ovarian tumors, except 2 in one animal, were granulosa cell tumors not unlike those described previously. The larger tumors ranged up to 2 cm in diameter. Three of the tumors contained large cysts. Two tumors in one of the mice receiving testosterone propionate were luteomas. Fourteen of the mice given testosterone propionate and 9 of the mice given sesame oil had bilateral tumors. Nine and 10 of the mice in the 2 groups, respectively, had large unilateral tumors. Most of the small tumors appeared near the hilus of the ovary. The impression was obtained that they arose from germinal epithelial ingrowths in this area. Ducts or cysts lined by a pseudostratified or columnar epithelium almost always occupied the hilus or ovarian stalk region but no evidence of their contribution to the tumors could be obtained. Occasionally these ducts were surrounded by the tumors. The ducts were also present in the ovaries of the estradiol-treated mice.

Histologically the non-tumorous ovaries of the testosterone-treated and control groups were very small—less than 1 mm in diameter—and contained ingrowths from the germinal epithelium and large clear or brown interstitial cells. Pituitary deficiency-cells were noted only in ovaries in mice in which the contralateral ovary was tumorous. The ovaries of the estrogen-treated mice were also small—less than 1 mm unless involved in leukemia—and consisted of multiple small anovular follicles obviously arising from the germinal epithelium and a cellular interstitial tissue showing nuclei with the peripheral chromatin aggregates characteristic of the gonads of hypophysectomized animals.

The lymphomas were not unlike those previously described in irradiated mice and ranged considerably in their histological structure and extent of involvement of other organs or tissues.

Three of the mice given testosterone propionate had subcutaneous fibrosarcomas and but one occurred in mice of each of the other 2 groups.

Summary and conclusion. Irradiated mice given estradiol benzoate did not have ovarian tumors whereas mice given testosterone propionate (1.25 mg weekly) or sesame oil did. Irradiated mice given testosterone propionate had fewer lymphomas than did the mice given estradiol benzoate or sesame oil.

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Comparative *in Vitro* Response of Tubercle Bacillus (H37RV) to Dihydrostreptomycin, Para-aminosalicylic Acid, and Dihydrostreptomycin-para-aminosalicylate.* (18223)

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The authors reported results of *in vitro* experiments(1) indicating that the two drugs streptomycin and PAS, when used together, were more effective in inhibiting the growth of tubercle bacilli than either drug used alone. This was also found to be true in *in vivo* studies(2). Hobby(3), studying a PAS salt of streptomycin consisting of 56 parts of streptomycin and 44 parts of PAS, reported that this compound was twice as effective as an equivalent amount of streptomycin used alone. She further reported early studies indicating that strains of tubercle bacilli resistant to the compound did not readily emerge, none having been isolated after a three-month period of trial. This is in sharp contrast to the well known fact that streptomycin-resistant organisms emerge quite rapidly both *in vitro* and *in vivo* when exposed to this drug, but in keeping with reported *in vitro* studies indicating that the presence of PAS together with streptomycin prevents the emergence of resistance(4). PAS-resistant strains of tubercle bacilli have been reported in patients who have received prolonged therapy(5). *In vitro,* however, such resistant strains emerge only after many months of exposure to PAS(6). Attempts to isolate either streptomycin-resistant or PAS-resistant strains from cultures repeatedly exposed to the two drugs used together have been unsuccessful.

The present report deals with a comparative study of the *in vitro* effects of dihydrostreptomycin-para-aminosalicylate, of dihydrostreptomycin and para-aminosalicylic acid used together in amounts equivalent to those present in dihydrostreptomycin-para-aminosalicylate and of para-aminosalicylic acid and dihydrostreptomycin, each used alone, both with regard to the *in vitro* growth inhibiting effect and to the development of resistance.

Method. The dihydrostreptomycin-para-aminosalicylate (DHSM-PAS) used was a chemical compound in which one mole of dihydrostreptomycin (DHSM) was combined with three moles of para-aminosalicylic acid (PAS). DHSM and PAS were used in a 1:3 molecular ratio in experiments in which the 2 drugs were used together. For convenience in this paper, the use of the 2 free drugs together will be designated by the symbol

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TABLE I. Growth of H37Rv in Dubos Tween-albumin Medium Containing Various Concentrations of DHSM,* DHSM-PAS,† and the Combination of DHSM and PAS.‡

Drug used	DHSM in μg/cc .82	.41	.205	.103	.05	.025	.013	Control
DHSM	0	2+	3+	4+	4+	4+	4+	4+
DHSM-PAS	0	0	2+	3+	3+	4+	4+	4+
DHSM + PAS	0	0	0	+	4+	4+	4+	4+

* DHSM = Dihydrostreptomycin.
† DHSM-PAS = Dihydrostreptomycin-para-aminosalicylate.
‡ PAS = Para-aminosalicylic acid.
The concentrations of DHSM-PAS and the combination of DHSM + PAS is given in terms of the DHSM content.

DHSM+PAS as opposed to DHSM-PAS which refers to the chemical compound. The virulent human strain of tubercle bacilli H37Rv was used as the test organism in the sensitivity tests and as the parent strain in attempts to isolate drug-resistant organisms. In the latter attempts, organisms were serially transferred into increasing concentrations of the drugs. The tube used for each successive transfer was that containing the highest concentration of drug still allowing growth. All sensitivity tests were performed in Dubos Tween-albumin medium to which the drugs were added aseptically. The experiments were set up in triplicate and the final results recorded are the average of the three tubes.

Results. The first studies were made to compare the *in vitro* effectiveness of DHSM, PAS, DHSM-PAS and DHSM+PAS. Table I shows the results of a typical experiment. It will be seen that the inhibiting concentration of DHSM was 0.82 μg/cc; the inhibiting concentration of DHSM-PAS was one containing 0.41 μg DHSM/cc while 0.205 μg DHSM/cc inhibited growth completely when PAS was added in a 1:3 DHSM:PAS molecular ratio. This relationship between the end points of DHSM alone, DHSM-PAS and DHSM+PAS was consistent in all experiments, though the specific end point itself varied slightly. The results would seem to indicate that while the compound (DHSM-PAS) was more effective than an equivalent amount of DHSM, the two drugs DHSM and PAS used together (DHSM+PAS) in concentrations theoretically present in DHSM-PAS were more effective than an equivalent amount of the compound itself. The full significance of this finding remains to be determined.

Results of studies on the emergence of tubercle bacilli resistant to DHSM and to DHSM-PAS are shown in Fig. 1. The fifth transfer of the organisms into DHSM containing Dubos medium resulted in a strain growing readily in 2048 μg DHSM/cc. In contrast, the fifth transfer of organisms into DHSM-PAS resulted in a strain growing readily in only 4.0 μg DHSM-PAS/cc. It was not until the tenth transfer that a strain was obtained which grew readily in 1000 μg DHSM-PAS/cc. When this DHSM-PAS-resistant strain was tested for its sensitivity to DHSM alone, to PAS alone and to DHSM and PAS used together (DHSM+PAS) it was found that they were resistant not only to DHSM-PAS but also to DHSM and PAS whether used alone or together. Organisms which grew readily in 1000 μg DHSM-PAS/cc were not inhibited by 2000 μg DHSM/cc, by 1000 μg PAS/cc or by 1120 μg DHSM/cc plus 880 μg PAS/cc.

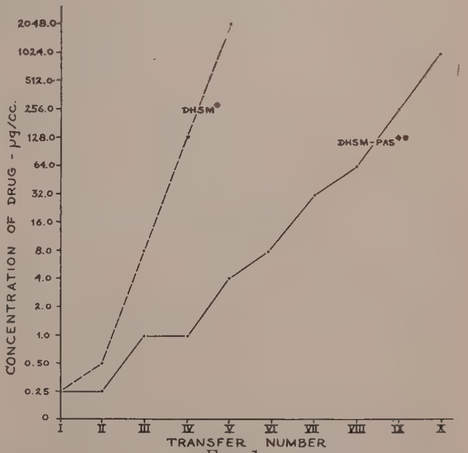

FIG. 1.

Emergence of resistance of H37Rv to DHSM and to DHSM-PAS as the result of serial transfers into increasing concentrations of each drug.
* DHSM = dihydrostreptomycin.
** DHSM-PAS = dihydrostreptomycin-para-aminosalicylate.

No increase in resistance has as yet been demonstrated by exposure of organisms to increasing concentrations of DHSM and PAS used together. These experiments are being continued.

Discussion. While it is recognized that *in vitro* results cannot necessarily be interpreted as *in vivo* effects, the *in vitro* difference between the results when a chemical compound (DHSM-PAS) was used as opposed to when the 2 drugs were added separately, make careful *in vivo* studies important. It had been hoped that the small amount of PAS which could be introduced into the streptomycin molecule might be sufficient to increase the effectiveness of the latter, as well as to reduce the important drawback of the rapid emergence of resistance. These experiments indicate that drug resistant organisms do emerge readily when a strain of tubercle bacilli is exposed to such a compound and that these organisms are resistant not only to the compound itself, but to both streptomycin and PAS as well.

Summary. 1. The *in vitro* growth of the H37Rv strain of tubercle bacilli was inhibited by 0.82 μg DHSM/cc, by a concentration of DHSM-PAS containing 0.41 μg DHSM/cc and by 0.205 μg DHSM/cc when PAS was added in a 3:1 molecular ratio of PAS:DHSM. 2. The fifth serial transfer of H37Rv into increasing concentrations of DHSM resulted in a strain growing readily in over 2000 μg DHSM/cc while the tenth such transfer of the organisms into increasing concentrations of DHSM-PAS resulted in a strain growing readily in over 1000 μg DHSM-PAS/cc. No increase in resistance on the part of organisms transferred into increasing concentrations of DHSM with PAS present in a 1:3 molecular ratio has so far been found. 3. Organisms resistant to the compound (DHSM-PAS) were found to be also resistant to DHSM alone, to PAS alone, and to the mixture of DHSM and PAS.

We are indebted to Mr. William Steenken, Jr., for the H37RV strain of tubercle bacilli and to the Ames Company for their generosity in supplying the dihydrostreptomycin para-aminosalicylate and the PAS used in these experiments.

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Genetic Aspects of the Added Carbon Dioxide Requirements of *Brucella abortus*.* (18224)

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It is well known that many strains of *Brucella abortus* require approximately 3 to 10% CO_2 in air for growth on primary isolation(1). However, some isolates lose the requirement for added CO_2 on continued subculture and may then be grown in air alone. The stability of the added CO_2 requirement of 9 strains of *B. abortus* was investigated as an initial step in the study of the role of CO_2 in the metabolism of members of this species.

Methods and cultures. Albimi brucella broth and agar were used as routine culture media and 1 ppm crystal violet was added to the agar for use in making plate counts. The diluent was an aqueous solution of 0.2% Difco tryptose-0.5% NaCl. CO_2 incubations were carried out in vacuum desiccators and the desired pCO_2 obtained by partial evacuation and replacement with tank CO_2. All incubations were at 38°C. Total counts were made using a Quebec colony counter and mutant counts were made using a dissecting microscope with transmitted light. Cultures

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* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1. Wilson, G. S., *Brit. J. Exp. Path.*, 1931, v12, 88.

TABLE I. Occurrence of Mutants Capable of Growth in Air in Cultures of *Brucella abortus* Krause.

Series	No. of cultures	No. of mutants	Cultures with no mutants	Total cells per culture
1	10	32	0	1.5×10^9
2	10	31	1	1.7×10^9
3	11	121	4	1.6×10^9
4	19	37	6	1.1×10^9
5	20	8	12	1.1×10^9
6	1	11	—	1.0×10^{10}

TABLE II. History and Mutation Rate for the Loss of Added CO_2 Requirement for Several Strains of *B. abortus*.

Strain	Source	Mutations per cell division $\times 10^{-10}$
Krause	Laboratory of Hygiene; human isolation	6.4
96251	Same	4.3
6232C	Camp Detrick; CO_2 stable	1.5
47257	Laboratory of Hygiene; human isolation	2.7
45095	Same	2.3
57087	Same	2.7
V-1	Univ. of Texas; guinea pig passage strain	3.2
335	Univ. of Texas; bovine udder isolation	3.9
1-SHL	Univ. of Texas; human isolation	3.2

were purified by single colony isolation. The sources of the strains employed in this investigation are listed in Table II.

Experimental. For the determination of mutation rate, flasks containing 100 ml broth were inoculated with 10^4 to 10^5 cells from a 24-hour culture grown under 10% CO_2. One ml of the inoculated broth was then transferred aseptically to each of 15 sterile (1.5 x 9 cm) tubes or 10 ml to each of 10 sterile Pyrex milk dilution bottles and incubated in a 10% CO_2-90% air mixture for 48 hours. Samples were taken from 2 or 3 cultures of each series for total counts which were determined by dilution and plating. These plates were incubated for 4 days in 10% CO_2. Mutant counts were determined by direct plating of whole 1 ml cultures or aliquots of the larger 10 ml cultures. These plates were incubated for 4 days in air. Results of a typical experiment are given in Table I. In each experiment several colonies from the plates incubated in air were transferred to agar slants. The mutants, which no longer require added CO_2 for growth, did not revert on serial subculture in either air or 10% CO_2. Cultures were tested for purity by Gram staining and high titer agglutination in antiserum against *B. abortus*. Both parent and mutant strains were smooth.

From the type of data given in Table I mutation rates could be calculated from the total number of mutants or from the fraction of cultures containing no mutants using the formulas of Luria and Delbrück(2) as modified by Lederberg(3). The data in Table II indicate very similar mutation rates for the 9 strains tested. The mean rate for this mutation is approximately 3 x 10^{-10} per cell division. A much greater variation in mutant counts was found in individual cultures than in replicate samples from the same culture, which indicates that the mutation is spontaneous and is not induced by the lower pCO_2 of air.

Calculation of mutation rate from the total number of mutants depends on similar growth rates of mutant and parent in mixed culture where the ratio of mutant to parent is very small. To test growth rates under these conditions a flask of broth was inoculated with the parent Krause stock and a mutant strain derived from it. The initial ratio of mutant to parent was $1.0{:}10^7$. After 48 hours incubation in 10% CO_2 the ratio was still $1.0{:}10^7$ although total counts had increased 10-fold. Growth rates of the two strains in pure culture when incubated in 10% CO_2 were identical during the logarithmic phase of growth.

It appeared possible that not all of the mutants were capable of forming colonies in the presence of large numbers of nonproliferating parent cells. To test this possibility a mutant strain from the parent Krause stock was plated in agar containing a background of 10^8 cells per ml of the Krause strain and in agar medium alone. The plates were then incubated in air. There was no significant difference in the counts of the two series, but

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2. Luria, S. E., and Delbrück, M., *Genetics*, 1943, v28, 491.

3. Lederberg, J., personal communication.

the colonies of the mutant were much smaller when plated in the presence of large numbers of parent cells.

Summary and conclusions. The results indicate that *B. abortus* loses its requirement for added CO_2 by spontaneous mutation at a rate of approximately 3 x 10^{-10} per cell division. Training of such cultures to dispense with their added CO_2 requirement probably consists of a selection of a spontaneously occurring mutant rather than gradual adaptation of the culture as a whole. The low mutation rate indicates a high degree of stability for CO_2 requirement in cultures of *B. abortus*.

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The Effect of Tocopheryl Esters on Influenza Virus. (18225)

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Miller and Dessert(1) have found that tocopheryl esters are capable of inhibiting the enzymatic depolymerization of hyaluronic acid by hyaluronidase, *in vitro*. Chemically there are certain similarities between hyaluronic acid (tissue mucin), glandular mucin and ovomucin. The latter two have been shown to behave as a substrate for the influenza virus enzyme. By competing in this capacity with the receptor substance of the red cell they prevent or inhibit* hemagglutination(2-5). The hemagglutination preventing property of ovomucin is gradually destroyed in the presence of live influenza virus, but is not affected by virus that has been heated 56°C for 30 minutes (in the case of PR 8). One type of hyaluronidase (pneumococcal) is also capable of altering ovomucin so that it no longer prevents hemagglutination by heated influenza virus(3).

The present paper undertakes to show that tocopheryl esters will also inhibit the enzymatic activity of influenza virus *in vitro*, and in addition are capable of lowering slightly the infectivity of the virus for chick embryos when the embryos are pre-treated with tocopheryl esters.

Materials and Methods. The PR 8 strain of influenza virus was used. The virus was propagated in 10-12-day-old chick embryos and stored in the form of frozen allantoic fluid. Ovomucin was prepared essentially by the method used by Gottschalk and Lind(5) The tocopheryl acid esters studied were the disodium salts of dl-alpha-tocopheryl phosphate (dl-alpha-Tph),† d-alpha-tocopheryl phosphate (d-alpha-Tph),‡ d-alpha-tocopheryl succinate (d-alpha-TS),‡ and a mixture of approximately equal concentrations of d-gamma-tocopheryl and d-delta-tocopheryl phosphates, referred to as d-delta, gamma-Tph.‡ Alpha-tocopherol itself was not studied because of the difficulty in obtaining a suitable solution. The tocopheryl esters were sparingly soluble in the 0.01 M phosphate buffer at pH 7.4, containing 0.85 per cent NaCl used as solvent and diluent for all reagents except

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* To avoid confusion the action of ovomucin on hemagglutination commonly called inhibition will, in this paper, be called prevention, and the term inhibition will be reserved for the action of the tocopheryls.

1. Miller, W. H., and Dessert, Alice M., *Ann. N. Y. Acad. Sciences,* 1949, v52, 167.

2. Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature,* 1947, v160, 404.

3. Hardy, P. H., and Horsfall, F. L., *J. Exp. Med.,* 1948, v88, 463.

4. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 442.

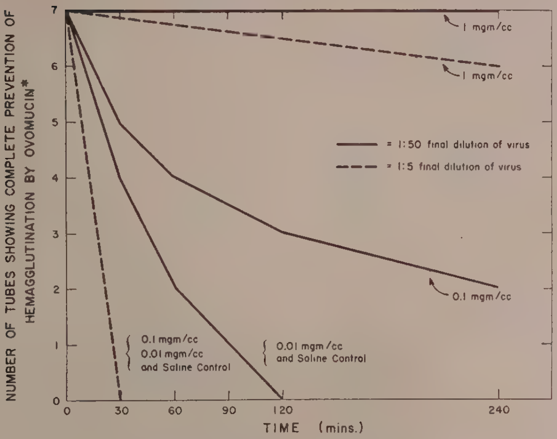
5. Gottschalk, A., and Lind, Patricia E., *Brit. J. Exp. Path.,* 1949, v30, 85.

† Hoffmann-LaRoche. A synethetic product.

‡ Distillation Products, Inc. Isolated from natural sources.

THE EFFECTIVENESS OF DIFFERENT CONCENTRATIONS OF dl-α-TPh AGAINST TWO DILUTIONS OF VIRUS

FIG. I (See Table I)



* It will be noted that by plotting the number of tubes instead of the actual dilution the ordinate is on a logarithmic scale. The other figures are also plotted this way.

where otherwise noted. The resulting solutions were close to neutrality.

In vitro tests. The amount of ovomucin present in a given solution was estimated in terms of the highest dilution at which the solution prevented hemagglutination by a given amount of heated PR 8 virus. Virus was added to a cold solution of ovomucin to which had been added either saline or a tocopheryl ester. The mixture was placed in a 37°C water bath. At appropriate intervals the tubes were shaken, samples withdrawn and immediately heated in boiling water for two minutes to destroy the virus present and stop the reaction. This amount of heating did not affect the ovomucin. To determine the residual amount of unchanged ovomucin, the sample was cooled and diluted serially 2-fold in saline (after an initial 1:10 dilution) in volumes of 0.4 cc. Eight to 16 hemagglutinating doses (HD) of virus previously heated to 56°C for 30 minutes were added in a volume of 0.2 cc. After 30 minutes at room temperature 0.2 cc of 0.5% suspension of thrice-washed chicken red blood cells (RBC) not more than five days old were added. The tubes were allowed to sit undisturbed at room temperature at least one-hour and then checked by the pattern technic(6) for hemagglutination prevention due to ovomucin. All experiments included a saline and ovomucin control (without virus) and a chicken cell agglutination (CCA) titer on the heated indicator virus dilution used. Tocopheryl esters in the concentrations used in these experiments did not affect the CCA titer of heated or unheated virus when the tocopheryl was added to the allantoic fluid (AF) and the fluid then diluted. In higher concentrations they caused hemolysis. When added to ovomucin solutions the tocopheryls caused an increased opalescence, but this did not affect the titration of the hemagglutination preventing property.

In vivo tests. The following procedure was used for the infectivity determinations: The required number of living 10-day-old chick embryos were inoculated via the allantoic cavity with the tocopheryl esters in various amounts, contained in volumes of 0.1-0.5 cc. An equal number of embryos were given an equal volume of buffered saline. By way of "sterilizing" the tocopheryl, penicillin was added to it and also in equal concentration to the saline inoculum so that each egg received about 250 units.[§] Both sets of embryos (treated and saline controls) were then inoculated intraallantoically with 0.1 or 0.2 cc

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6. Salk, J. E., *J. Immunol.*, 1944, v49, 87.

TABLE I. Effect of Various Concentrations of DL-alpha-Tph on Ability of PR8 to Destroy Hemagglutination-prevention Properties of Ovomucin. Ovomucin, 3 cc.

Vol. of reagents in order of addition		Time (min.)	Ovomucin hemagglutination-prevention titer									
			Tube No. 1	2	3	4	5	6	7	8	9	
1 cc of DL-alpha-Tph	1 cc of PR8		Dilution 10	20	40	80	160	320	640	1280	2560	C*
1 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0†
		240	0	0	0	0	0	0	±	+	+	0†
0.1 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
0.01 mg per cc	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
0 (1 cc saline)	1:5	0	0	0	0	0	0	0	0	+	+	0
		30	+	+	+	+	+	+	+	+	+	0
1 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0†
		240	0	0	0	0	0	0	0	+	+	0†
0.1 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	0	±	+	+	+	0
		60	0	0	0	0	±	+	+	+	+	0
		120	0	0	0	+	+	+	+	+	+	0
		240	0	0	+	+	+	+	+	+	+	0
0.01 mg per cc	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	±	+	+	+	+	0
		60	0	0	±	+	+	+	+	+	+	0
		120	+	+	+	+	+	+	+	+	+	0
0 (1 cc saline)	1:50	0	0	0	0	0	0	0	0	+	+	0
		30	0	0	0	0	±	+	+	+	+	0
		60	0	0	±	+	+	+	+	+	+	0
		120	+	+	+	+	+	+	+	+	+	0

* C = Control—no indicator virus.
† Slight hemolysis.
16 HD per tube.
+ = Hemagglutination.
0 = No hemagglutination.
The figure in the DL-alpha-Tph and PR8 columns represent final concentration and final dilution, respectively.

of the same serial dilution of a stock pool of PR 8 (allantoic fluid) stored frozen in small amounts in stoppered vials. Four to five dilutions were inoculated straddling the I.D.$_{50}$ endpoint, using eight eggs per dilution per set. The shell holes were sealed with paraffin. The eggs were then placed in a 35° incubator, candled at 24 hours for viability and at 48 hours removed to a cold room (4°C) overnight. The allantoic fluids were harvested and each fluid tested for hemagglutination by mixing equal volumes of 0.25% RBC and AF. Hemagglutination was taken to indicate infection. The I.D.$_{50}$ endpoints were calculated by the method of Reed and Muench(7). The allantoic fluids from the treated embryos often showed a noticeable opalescence. This was present in both the infected and non-infected embryos and was found to be considerably less or completely absent when fluids were harvested without previous chilling of the embryo.

Results. In Fig. 1 and Table I are presented the results of an experiment employing various concentrations of dl-alpha-Tph, two concentrations of virus and a constant amount of ovomucin. The undiluted allantoic fluid had a CCA titer of 1:2560. In this experiment the 10^{-1} dilution was made with the same virus-containing allantoic fluid heated 65°C for 30 minutes to destroy the virus. This was done to make certain that the effects obtained when the virus was diluted were due to virus dilution alone and not to dilution of some non-specific (*e.g.* mineral) factor in the AF. When 1 cc of undilute AF was added to the

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§ When streptomycin (calcium salt) was mixed with the tocopheryl a heavy precipitate formed. It was not used even though there were indications that the tocopheryl was still active when injected.

7. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

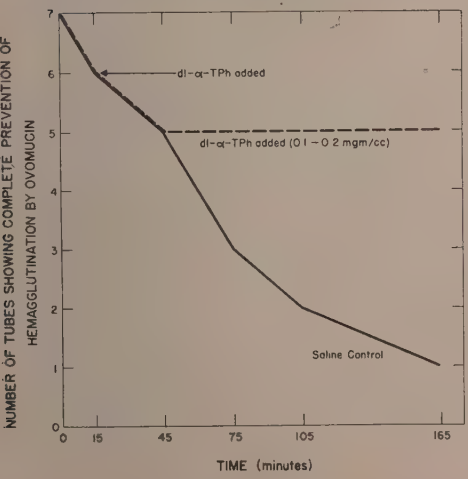
ovomucin-tocopheryl the final dilution became 1:5, and similarly for 10^{-1} the final dilution became 1:50. It can be seen that with the substrate (ovomucin) constant the degree of inhibition by tocopheryl was directly related to the amount of tocopheryl present and inversely related to the amount of virus.

Corollary experiments varying the concentration of substrate were not done. Increasing the ovomucin concentration (without concentrating the virus) necessitated prolonged incubation periods which would have allowed too much opportunity for non-specific factors to come into play. Decreasing the substrate concentration by a significant amount made for insufficient spread between the initial and final titrations.

It was then determined whether the tocopheryl ester could exert its effect after the virus-substrate reaction had already been initiated. Virus and ovomucin were mixed and placed in a 37°C water bath. Samples were taken at 0 time and at 15 minutes. Then the virus-ovomucin mixture was divided equally among three tubes containing respectively saline and dl-alpha-Tph in sufficient quantity to make final concentrations of 0.1 and 0.2 mg per cc. In the control tube ovomucin continued to be destroyed, whereas in the tubes containing dl-alpha-Tph the ovomucin titer fell at first then leveled off. Fig. 2 illustrates these results. The 4 tocopheryl esters that were available were then compared. All were tested at a concentration of 0.1 mg per cc, and were always run simultaneously against various concentrations of virus. Fig. 3 gives the results with 1:50 final dilution of virus. Dilutions of 1:25, 1:37.5, 1:62.5 and 1:75 gave the same relative results; d-alpha-Tph and dl-alpha-Tph were most effective, d-alpha-TS somewhat less so, and d-delta, gamma-Tph hardly at all.

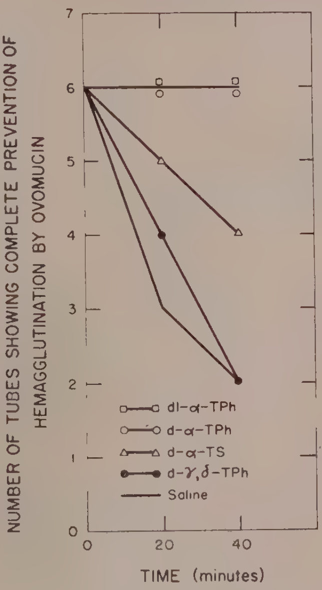
EFFECT OF ADDITION OF dl-αTPh AFTER PR8-OVOMUCIN REACTION HAS BEGUN

FIG. II



RELATIVE EFFECTIVENESS OF VARIOUS TOCOPHERYLS AGAINST 1:50 VIRUS

FIG. III



The following experiments were devised to determine whether the action of the tocopheryl esters was reversible or whether it induced a permanent change in the virus that persisted when the virus was removed from the esters. Allantoic fluid containing PR 8 was treated with dl-alpha-Tph and the virus was then precipitated by cold methanol and recovered according to the method of Cox, *et al.*(8). An untreated control was similarly manipulated.

The addition of dl-alpha-Tph to the un-

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8. Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, v56, 149.

TABLE II.
Chick Embryo Infectivity Experiments.

A.

Exp. No.	Treatment	mg per egg	Log I.D.$_{50}$
E-10	Saline	—	8.3
	DL-alpha-Tph	1	7.5
11	Saline	—	8.4
	DL-alpha-Tph	1	7.6
	DL-alpha-TS	1	7.5
13	Saline	—	8.3
	D-alpha-Tph	1	7.7
	D-delta, gamma-Tph	1	8.1

B.
10^{-8} Dilution

Controls		Treated	
Positive by h*	Negative by h*	Positive by h*	Negative by h*
21	11	9	23

*h = Hemagglutination.

diluted AF to a final concentration of 1 mg per cc did not cause a noticeable decrease in viral activity. The virus recovered by precipitation was also active. When the concentration of dl-alpha-Tph was increased to 2 mg per cc the viral enzyme activity was inhibited both before and after recovery by precipitation and there was also a decrease in recoverable virus. These experiments, however, were considered inconclusive.

Chick embryo infectivity experiments: Table II-A gives a summary of 3 infectivity experiments performed as outlined above, using a dose of 1 mg per egg of tocopheryl. Table II-B represents the combined results at the 10^{-8} dilution for the 3 experiments (not including d-delta, gamma-Tph). When evaluated by the Chi squared method using the Yates modification for small numbers and by "Student's" "T test" these differences are significant. In those treated eggs in which infection occurred, the CCA titer was equal to that of the controls, the virus was active in breaking down ovomucin and on further passage caused infection. Further passage of some of the fluids from the treated, non-infected embryos did not cause infection at dilutions of 10^{-0} and 10^{-3}. In experiments employing doses of 0.5 mg per egg the effect was smaller. Doses of 2 mg per egg were toxic.

It will be noted that d-delta, gamma-Tph had little if any effect. This corresponds with the *in vitro* findings. The relative effectiveness of these compounds *in vitro* and *in vivo* against influenza virus are the same as found by Miller and Dessert(1) against hyaluronidase *in vitro*.

Discussion. The possible modes of action of the tocopheryl esters upon influenza virus are many and for a discussion of the action of these compounds upon other enzyme systems, the reader is referred to(9), especially Part II. The experiments reported herein, however, appear to indicate some specificity for the alpha configuration against influenza virus enzyme. It is not immediately apparent why, if the activity of the tocopheryl esters against *in vitro* enzyme systems are due to the protein precipitant or detergent-like or metal-ion combining properties conferred upon them by the phosphate radical, the same activity is not exhibited by the phosphate esters of delta and gamma tocopherol. This same question was raised by Miller and Dessert(1) with regard to hyaluronidase. The other enzyme systems discussed in(9) appear to be inhibited equally as well by the delta and gamma tocopheryl esters as by the alpha configuration.

It would seem worth while to prepare other tocopheryl esters and examine their activity against influenza virus.

Summary. Alpha-tocopheryl esters are capable of inhibiting the enzymatic activity of influenza virus *in vitro* and of protecting chick embryos against infection by small doses of virus. A mixture of delta and gamma tocopheryl esters was not effective.

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9. Symposium on Vitamin E., *Ann. N. Y. Acad. Sciences,* 1949, v52, 63.

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Observations on Aureomycin and APF for the Pig. (18226)

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(Introduced by Paul B. Pearson)

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Jukes *et al.*(1) showed that aureomycin would produce a growth response with the pig when added to diets containing B_{12} and B_{12b}. This increase in rate of growth was approximately equal to that obtained from supplementation with an aureomycin fermentation product designated APF (Lederle).

In the experiment reported herein, Series 1 was conducted to determine the effect of aureomycin supplementation over a longer period than was previously reported by Jukes *et al.*(1). Series 2 was conducted to determine whether APF Supplement 2 contains a growth promoting factor(s) besides aureomycin and B_{12} and to determine if supplementation with aureomycin in addition to APF Supplement 2 would result in a further increase in the rate of gain.

Materials and methods. The basal ration used in both series consisted of ground yellow corn 57, peanut meal (hydraulic) 41.5, bone meal 0.5, limestone 0.5 and salt-trace mineral mixture 0.53 lb (the salt-trace mineral mixture consisted of iodized salt 50 lb, manganese sulfate 951 g, ferrous sulfate 398 g, copper sulfate 125 g and cobalt carbonate 10 g). Vitamins A and D and the seven B-complex vitamins which the pig has been shown to need in the ration were added at levels previously described by Cunha *et al.*(2). In Series 1, 4 purebred Duroc pigs were fed in each lot. Five grade native pigs (of mixed breeding and which had been depleted on a maintenance ration since weaning) were fed in each lot in Series 2. The pigs were fed on concrete floors which were washed once daily. Series 1 was conducted for 54 days and Series 2 for 67 days.

Results and comments. The results obtained in Series 1 show that aureomycin supplementation considerably increased the rate of gain. During the 37-day trial, the higher level of aureomycin (lot 3) was more beneficial in stimulating growth than the lower level (lot 2). The optimum level of aureomycin is not known yet and it may differ depending on the kind of basal ration used.

In both series, the pigs fed aureomycin and those on APF Supplements 1 and 2 had considerably more bloom and a smoother hair coat than the pigs on the basal ration plus B_{12}. The hemoglobin values were higher with the pigs fed either aureomycin or APF Supplement 1 (Series 1).

In Series 2, supplementation of aureomycin in addition to 1% of APF Supplement 2 (lot 2) in the ration did not increase the rate of growth over that obtained with 1% of APF Supplement 2 in the ration (lot 3). This shows that 1% of APF Supplement 2 in the ration supplied enough aureomycin for the pig. The supplementation of the basal ration with 3% of APF Supplement 2 (lot 4) caused an increase in rate of gain over that obtained with 1% of APF Supplement 2 or with 1% of APF Supplement 2 plus aureomycin. Therefore, the increase in rate of gain caused by 3% of APF Supplement 2 indicates that some factor(s) other than aureomycin and B_{12} in APF Supplement 2 benefited growth for the pig. Whether APF Supplement 2 was supplying some unknown factor(s) or some known factor which might be low in the basal ration remains to be determined. The level of APF Supplement 2 which will adequately supply the needs of the pig for aureomycin has not yet been determined but a level of 1% supplied enough aureomycin under the conditions of this trial. At the level APF Supplement 2 was added in lot 3 it was supplying 60 mg of aureomycin per kilo of diet.

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TABLE I. Series 1—Effect of Aureomycin Supplementation.

Lot No.	Avg initial wt in lb.	Supplement/kg basal diet	Avg daily gain in lb. first 37 days	Avg daily gain in lb. total 54 days	Hb.§
1	35.6	44 γ vit. B_{12} (as concentrate)†	0.77	0.75	10.7
2	35.8	44 γ vit. B_{12} + 50 mg aureomycin	1.30	*	—
3	35.6	44 γ vit. B_{12} + 200 mg aureomycin	1.61	1.69	13.1
4	35.6	10 g APF‡	1.36	1.46	12.0

* Lot 2 was stopped after being on experiment 37 days.

† Vit. B_{12} concentrate contained approximately equal parts of vit. B_{12} and B_{12b} and had at least 10 mg of vit. B_{12} activity per g of solids.

‡ Supplement 1 was a commercial APF supplement (8108-109) prepared from the aureomycin fermentation at Lederle Laboratories. By microbiological assay, Supplement 1 was found to contain about 3 mg of aureomycin per g. Its B_{12} content by chick assay appears to be about 4 μg per g. The APF, aureomycin and B_{12} were obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

§ Average of hemoglobin at end of 54 days.

TABLE II. Series 2—Effect of Aureomycin and Supplement 2.

Lot No.	Avg initial wt in lb.	Supplement/kg basal diet	Avg daily gain in lb., 67 days
1	42.5	1.5 g methionine* + 27.5 γ vit. B_{12}†	0.76
2	41.7	1.5 g methionine + 10 g APF‡ (1%) + 100 mg aureomycin‡	1.26
3	44.5	1.5 g methionine + 10 g APF (1%)	1.26
4	44.8	1.5 g methionine + 30 g APF (3%)	1.56

* DL-methionine supplied through the courtesy of Dr. H. J. Prebluda, U. S. Industrial Chemicals, New York City.

† Vit. B_{12} concentrate (fuller's earth) from Dr. D. F. Green, Merck, Rahway, N. J.

‡ Supplement 2 (lot 24) was similar to Supplement 1 except that microbiological assay indicated its aureomycin content was 6 mg per g.

Summary. Aureomycin supplementation produced considerable growth response with the pig when added to a diet containing vitamin B_{12} and B_{12b}. APF Supplement 2 contains some factor(s) other than aureomycin and B_{12} which accelerates growth in the young pig with the basal ration used. One per cent of APF Supplement 2 in the ration supplied enough aureomycin for the pig under the conditions of this experiment.

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Desoxycorticosterone Hypertension and Ammonium Chloride.* (18227)

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Prolonged administration of desoxycorticosterone acetate (DCA) results in the development of hypertension in a variety of animal species including man(1-4). In the rat, this is accompanied by a condition of widespread arteriolosclerosis, nephrosclerosis, periarteritis nodosa and cardiac lesions(1,3,5). Increased sodium chloride intake may augment both the hypertension and the severity of the lesions(5,6), but does not apparently do so when high doses of DCA are employed (6). Although a sodium free diet prevents these effects of DCA in rats(7), clinical evidence suggests that sodium retention is not a factor in the increased blood pressure which develops in either Addisonian(4) or hypertensive patients(8) receiving DCA. The relationship which exists between DCA-induced hypertension and sodium therefore is not entirely clear. Ammonium chloride, administered to rats receiving DCA, inhibits the widespread cardiovascular lesions(9,10). This has been ascribed to the prevention of the hypertensive effect of the hormone(9,11), although the blood pressure was not measured; the deduction being made from the inhibition of cardiovascular lesions. Further, although the beneficial effect of ammonium chloride has been ascribed to its sodium diuretic effect, there is some doubt as to whether blood sodium may be effectively lowered by ammonium chloride(12,13). Evidence indicates that hypertension resulting from DCA treatment precedes the development of cardiovascular lesions(14), and may persist even when DCA is withdrawn(15). The foregoing considerations suggested to us that it might be well to re-evaluate the influence of ammonium chloride on DCA hypertension.

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* This study was supported by a grant from the American Heart Association.

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Methods. Sixty albino rats of the Holtzman strain, half females and half castrate males, weighing between 40 and 65 g were unilaterally nephrectomized and divided into 6 equal groups. Groups 1 and 2 received a solution containing 0.5% sodium chloride to drink: groups 3 and 4 were given a solution of ammonium chloride containing an equivalent number of chloride ions, *i.e.* 0.45% NH_4Cl: groups 5 and 6 received a solution which contained 0.5% NaCl and 0.45% NH_4Cl. A pellet of crystalline desoxycorticosterone acetate† weighing 50 mg was implanted subcutaneously into each of the rats in the odd numbered Groups 1, 3 and 5; whereas those of Groups 2, 4 and 6 served as controls. All of the solutions were made with distilled water. The food consisted entirely of Purina Laboratory Chow. On the first day of the experiment all animals were allowed free access to drinking fluids, and it was found that those of Group 4 consumed the least. Thereafter the animals of this group were allowed fluid *ad lib.*, and the other groups were restricted to the amount which Group 4 voluntarily consumed. The blood pressures were taken, beginning on the 15th day of treatment, twice weekly for the duration of the experiment by the tail plethysmograph method of Williams, Harrison and Grollman(16), as modified by Sobin(17), on unanesthetized animals. In order to avoid bias one of us (L.A.P.) measured the blood pressure once weekly while another (O.H.) recorded the pressures at the second weekly determination. On each of these occasions the operator measured the pressures while being unaware of the group to which the animal in question belonged or of the previous pressures determined in that week. An average of 4 successive readings on each rat was taken to be an accurate representation of the

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† We are indebted to the Schering Corp., Bloomfield, N. J. for supplying the desoxycorticosterone acetate used in these studies.

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blood pressure in that animal on each occasion. On the 30th day the animals were sacrificed and the kidneys, hearts, adrenals and portions of the pancreas and mesentery were placed in Bouin's fixative. The auricles were removed from the hearts at the time of autopsy since we have found ventricular weights to be a more sensitive reflection of cardiac hypertrophy than total heart weights, and organs were weighed after fixation.

Results. In our experience normal rats do not exhibit blood pressures in excess of 135 mm Hg, which therefore represents the upper limit of normal. Pressures of 150 mm Hg or higher have arbitrarily been designated as reflecting a hypertensive state. The blood pressure of the rats in Groups 2, 4 and 6 did not deviate from the normal at any time during the course of the experiment. Those receiving DCA however began to manifest hypertensive pressures about the 19th day of treatment in Groups 1 and 3, and those of Group 5 about the 23rd day. Actually those of Group 3, receiving ammonium chloride and DCA, had blood pressures slightly higher than those of Groups 1 and 5. Although this difference between Groups 1 and 3 was not statistically significant at the final reading, the blood pressure of the animals in Group 5 was significantly lower ($P<0.02$) than that of Groups 1 and 3. The rats of Group 5 lagged behind those of the other DCA treated groups, both in the time at which the elevation of blood pressure became manifest and the magnitude of the elevation (Fig. 1). The reason for this is not readily apparent although the rats of Groups 5 and 6, while they appeared to be in good condition, did not gain as rapidly in weight as the other rats in this experiment, perhaps because they received a hypertonic solution to drink.

Since the rats in Group 3 did not consume their fluid at a much greater rate than those of Group 4, these groups, for all practical purposes, had fluid available at all times. Those of Group 2 consumed their daily allotment within 4 or 5 hours; those of Group 1 within an hour; and those of Groups 5 and 6 in about 8 and 12 hours respectively. At the beginning of the experiment the average fluid intake of each rat was 14 cc; whereas terminally it reached 25 cc daily.

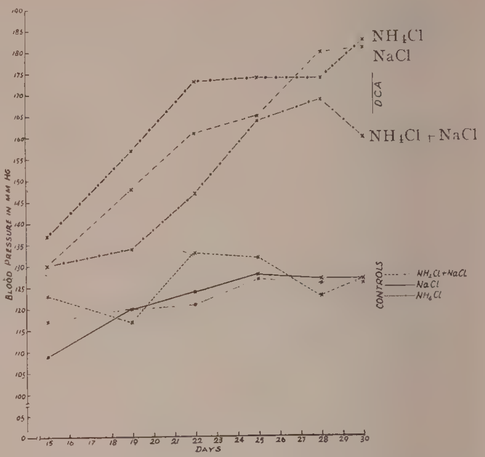

FIG. 1.

Blood pressure of DCA-treated and control rats. The slope of the curve for rats receiving the hormone and ammonium chloride is paralleled, at a somewhat lower level for the greater part of the experiment by that of the rats receiving DCA and sodium chloride. Hypertension in rats receiving DCA and both electrolytes is delayed in development and of lesser magnitude. Days on which blood pressure determinations were made are indicated on the chart by the letter X.

At the end of the experimental period every rat in Groups 1, 3 and 5 was hypertensive. The organ weights, together with their standard errors calculated according to the formula

$$\text{S. E. M.} = \sqrt{\frac{d^2}{n\,(n-1)}},$$

are given in Table I. The effect of DCA in producing cardiac and renal enlargement is apparent in each of the groups receiving the steroid. Similarly the weight of the adrenals was depressed in those animals receiving DCA, below that of the appropriate controls, this difference not being marked between Groups 1 and 2. This may perhaps be due to the fact that being deprived of drinking fluid for about 23 hours of the day proved a sufficient stress to permit the pituitary to elaborate normal amounts of ACTH despite the mild inhibitory influence of DCA on this function of the hypophysis.

Histologic study of the tissues examined failed to reveal pathologic lesions in any of

TABLE I. Effect of Desoxycorticosterone Acetate on the Body and Organ Weights of Rats Drinking Various Electrolyte Solutions.

Drinking solution	NaCl		NH_4Cl		NaCl and NH_4Cl	
Group	I DCA	II Control	III DCA	IV Control	V DCA	VI Control
No. of rats (final)	9	7	8	10	7	8
Body wt (g)						
Initial	51 ± 2	46 ± 1	54 ± 2	51 ± 2	43 ± 1	48 ± 5
Final	120 ± 4	132 ± 4	127 ± 1	140 ± 3	111 ± 1	112 ± 4
Kidney wt						
mg	1100 ± 30	953 ± 13	1127 ± 67	899 ± 38	919 ± 64	807 ± 48
% body wt	.921 ± .028	.688 ± .011	.862 ± .041	.639 ± .084	.812 ± .031	.715 ± .011
Heart wt						
mg	444 ± 17	420 ± 19	493 ± 10	354 ± 21	393 ± 9	354 ± 21
% body wt	.368 ± .009	.317 ± .017	.398 ± .023	.282 ± .007	.349 ± .011	.282 ± .007
Adrenal wt						
mg	24 ± 1	25 ± 1	24 ± 1	27 ± 1	19 ± 0	22 ± 0

the rats. This may be due to the low dosage of DCA and short duration of the experiment. Also the rather rigorous restriction of fluid, and hence electrolytes, perhaps militated against their development.

Discussion. Rats receiving desoxycorticosterone acetate develop a marked and persistent hypertension against which ammonium chloride exerts no protective effect. The elevation of blood pressure to hypertensive levels occurs as promptly and as markedly in rats receiving ammonium chloride to drink as in those given comparable quantities of sodium chloride; indeed, as may be observed from a perusal of Fig. 1, the blood pressure of the former group was at all times slightly above that of the latter. Hypertension produced by the steroid in rats which received a hypertonic solution of sodium and ammonium chloride together was slightly delayed in time of onset and was of lesser magnitude. It has been shown that ammonium chloride does not interfere with the life maintaining action of DCA in the adrenalectomized rat(18). The present study would indicate that this electrolyte similarly does not interfere with the hypertensive effect of the steroid. Perhaps rats allowed free access to unlimited amounts of sodium chloride and treated with DCA might have developed a more severe hypertension than those on restricted quantities, but it is evident that when sodium chloride intake is limited it does not potentiate the pressor effect of DCA to a greater extent than equivalent amounts of ammonium chloride.

The inhibition of cardiovascular lesions in DCA treated animals by ammonium chloride would therefore seem to be due to a local effect on the tissues involved rather than to any interference with the production of hypertension *per se*. This would appear to be of considerable therapeutic interest since although cardiovascular pathology is an almost invariable consequence of sustained hypertension, any form of therapy which fails to reduce the blood pressure is usually discarded. Ammonium chloride, while failing to reduce the blood pressure of DCA treated animals, may nonetheless be effective in moderating the severity of the widespread vascular damage which follows sustained hypertension.

Summary. Ammonium chloride does not inhibit the hypertensive effect of desoxycorticosterone acetate in the rat. When both received the steroid, no difference can be detected between the blood pressure of rats given excess sodium chloride or an equivalent amount of ammonium chloride. The protection against cardiovascular lesions by NH_4Cl in DCA treated animals must therefore be ascribed to a lack of response of the vascular territories to hypertension.

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Histologic Findings in Rats Subjected to Prolonged Administration of Para-Aminobenzoic Acid. (18228)

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The biologic importance of para-aminobenzoic acid (PABA) has been recognized only recently. Its first important clinical use, stemming from the work of Snyder and his colleagues(1), was for the treatment of epidemic typhus fever(2). During World War II most of the other major rickettsial diseases were shown to be favorably modified by the drug. More recently PABA has been found to benefit a variety of chronic disorders, including scleroderma(3), dermatomyositis(3), certain forms of lupus erythematosus(4), lymphoblastoma cutis, and dermatitis herpetiformis. In contrast to the relatively brief treatment necessary in the rickettsial infections, these chronic conditions may require the continuation of therapy with PABA for weeks, months, and perhaps indefinitely. While previous studies have indicated that PABA has a low degree of toxicity in acute or short-term experiments(5), these recent clinical applications raise the question of possible greater toxicity in prolonged administration of the drug. Our interest in the problem was enhanced by the observation at autopsy of severe retrogressive changes in the hepatic cells in one of many patients treated with PABA for lupus erythematosus(4). For these reasons an investigation was undertaken to determine the toxicity of PABA in long-term feeding experiments with rats. The gross and microscopic findings of that study form the basis of the present report.

Method. Young inbred albino rats weighing 50 to 70 g were divided into 3 groups of 10 animals each. All were fed pulverized Purina fox chow checkers and trimmings from green vegetables. Drinking water was accessible at all times. Males and females were not segregated, except for short periods following the birth of young. At such times the female and her litter were caged separately until after weaning. The rats of Group I served as controls. Group II received the sodium salt of PABA (sodium para-aminobenzoate) and Group III, the corresponding potassium salt. The drugs were administered in the diet by adding to pulverized chow pellets a measured amount of the respective powdered compound or of a stock 10% solution of drug. Sufficient water was added to give the mash a moist, porridge-like consistency for each of the 3 groups. The food-drug mixture was prepared fresh every day and the animals offered an amount barely in excess of their average total intake. The quantity of drug employed was adjusted to provide a daily dosage of 2 g per kilo of body weight. PABA levels were determined on samples of blood drawn from the heart at various intervals after feeding. The Marshall and Litchfield(6) method for sulfonamides was adapted for this purpose. From time to time urine specimens were pooled from each group and tested with Benedict's reagent for reducing substance. Two animals of each group were killed by etherization after intervals of 1, 2, and 3 months respectively. The remainder were sacrified after the drug had been ingested for 6 months. On each animal a complete autopsy was performed immediately after death, and tissues were fixed in 10% formalin. Specimens of liver were fixed also in absolute alcohol for glycogen stains. All were imbedded in paraffin, and sections of brain, pituitary gland, heart, lungs, salivary glands, thyroid gland, thymus, spleen, stomach, intestines, pancreas, liver, adrenals, kidneys, and testes (or uterus, tubes, and ovaries) were stained with hemalum and eosin. In addition frozen sections of heart, lungs, liver, adrenals, and kidneys were stained for neutral fat with Sudan IV. Sections of alcohol-fixed liver were stained by Best's carmine method for glycogen.

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Results. There were observed no significant differences between the treated and untreated animals with respect to body weight, rate of growth, and organ weights. There was likewise no apparent interference with reproduction, as approximately the same number of normal litters were born in each group. That the PABA was adequately absorbed is evident from the blood level determinations, which ranged from 2.0 to 7.0 mg % during the first 6 hours after feeding and from 0.2 to 2.6 mg % up to 24 hours later. Urine of the treated animals occasionally revealed traces of reducing substance.

Histologically, the only significant changes noted in the treated animals were in the thyroid glands. These alterations consisted of reduction in the amount of intrafollicular colloid and decrease in the intensity of its eosinophilic staining reaction. There was an associated increase in the number and height of the epithelial cells lining the affected acini. In general, the involvement was most marked in the central regions of the gland, but similar changes of a lesser degree were present in peripheral portions. These effects were maximal after the administration of PABA for 2 months; thereafter they became progressively less conspicuous, although still present in the animals treated for 6 months.

Aside from the changes in the thyroid glands, no significant abnormalities were observed in the sections of any tissue or organ, whether stained routinely or by the special methods for fat and glycogen.

Discussion. The "goitrogenic" action of PABA has been well documented previously, and it seems necessary, therefore, only to point out that the thyroid changes in these animals conformed to those of earlier investigations(7-9). The possibility of a hepatotoxic action of PABA is of particular interest and of obvious clinical importance. Scott and Robbins(10) reported acute necrosis of the liver in dogs given massive oral doses of the drug (2 g per kilo at one time.) In this connection, it appears significant that the liver is believed to be the primary site of "detoxification" of PABA as well as other aromatic amines(11). While much PABA is eliminated in the "free" form, some undergoes "detoxification" in the liver by acetylation of the amino group and some by conjugation with glucuronic acid(11). The presence of the latter conjugate has been almost uniformly observed in the urine of patients under treatment with large doses of PABA(12). The finding of traces of reducing substance in the urine from these rats is presumably due to the same compound. Moreover it has been suggested that the glucuronic acid utilized in this conjugation process is derived from hepatic glycogen(13). With these points in mind it was anticipated that marked diminution in liver glycogen, and perhaps other hepatic changes, might be produced. However, no hepatic lesions were observed.

Preliminary studies of the effects of PABA upon chronically starved rats have likewise revealed no evidence of hepatic damage, although a marked depletion of liver glycogen was observed in both treated and untreated starved animals.

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Summary. 1. In doses greatly exceeding the usual therapeutic range for man, para-aminobenzoic acid is a relatively non-toxic substance for the rat. 2. Aside from histologic alterations in the thyroid gland, no significant pathologic changes were detected in the treated animals or in their offspring. 3. The thyroid changes consisted of reduction in the amount and eosinophilia of intrafollicular colloid, associated with epithelial hypertrophy and hyperplasia. These findings were maximal after 2 months of treatment. 4. No hepatotoxic effect was detected.

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Electrical Injury of the Cochlea of the Guinea Pig.*† (18229)

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The original purpose of the present experiments was to polarize electrically the sense organ of the inner ear in the expectation that the thresholds of the aural microphonic and of the action potentials and perhaps also their maximum amplitudes would be modified. Any such modifications would be useful tools for analysis of the origin of the microphonic and of the excitation of the primary neurons by the sensory cells. The method seemed practical because of the success of Riesco and collaborators(1) in drilling small holes into the cochlea of the guinea pig and thereby placing electrodes in known positions relative to the basilar membrane. Our expectations were fulfilled and the results will be presented elsewhere. The present report describes injurious effects produced incidentally by electro-coagulation and electrolysis in our exploratory experiments.

Methods. Holes were drilled as described elsewhere(2) into at least 2 and sometimes 3 turns in the cochlea and another electrode was regularly placed on the round window. No. 38 enamel-insulated silver wire was substituted for the original copper wires. The usual positions of electrodes and their attachment by dental cement to the edge of the opening into the acoustic bulla is shown in Fig. 1. Frequently, however, the electrode in turn 1 was introduced into the scala tympani instead of scala vestibuli by placing it on the opposite side of the dark band that marks the position of the stria vascularis. The thresholds of the aural microphonic at frequencies 200, 1000, 2500 and 8000 cps and also of the action potential in response to a standard filtered click were measured(2). The par-

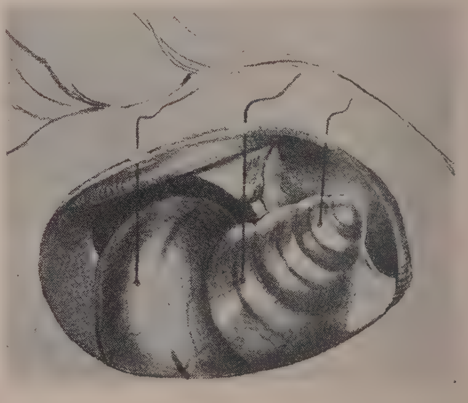

FIG. 1.

Three silver wires (black lines) are placed as electrodes in the first, second and fourth turns of the cochlea of a guinea pig. The wires are held in place by dental cement attaching them to the cut edge of the bulla. The dark band that marks the position of the stria vascularis is also shown.

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* This work was carried out under Contract N6onr-272 between the Office of Naval Research and Central Institute for the Deaf.

† Presented in part at the meeting of the Missouri Section, March 22, 1950.

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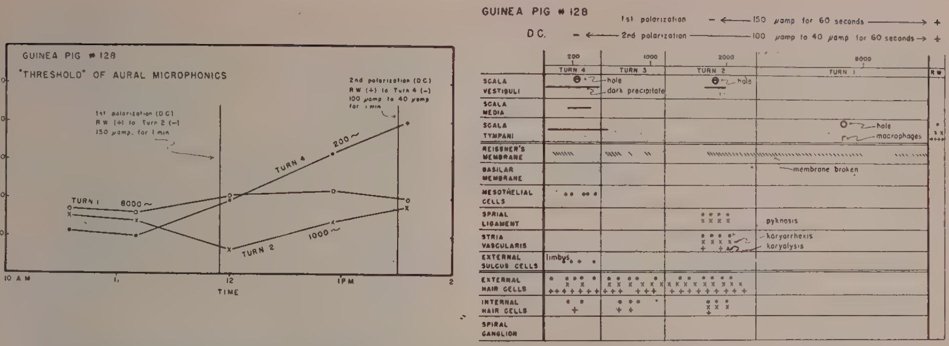

FIG. 2.

Effects of DC; 2 doses of 1 minute each between electrodes as indicated. Injury centering in Turns 4 and 2. Considerable rise in threshold for low tone. Little effect on 8000 cps or 1000 cps. (Turns 1 and 3 are less seriously injured).

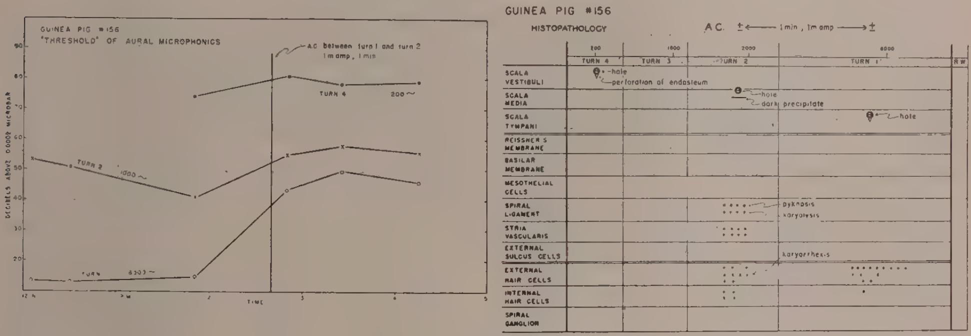

FIG. 3.

Effects of AC; 1 milliampere for 1 minute. Moderate injury in Turns 1 and 2. Great rise in threshold for 8000 cps, slight for 1000 cps (Turn 3) and 200 cps (Turn 4).

ticular test frequencies were chosen because the positions on the basilar membrane which they agitate most strongly correspond to the positions of the electrodes shown in Fig. 1.

A DC electric potential of known strength and polarity was applied between 2 electrodes. In a later series of experiments alternating current at 1000 cps or radio-frequency current at 4.0 megacycles was employed. Sometimes the two polarizing electrodes were located in the same scala but in different turns so that the potential drop was lengthwise of the basilar membrane. More often the basilar membrane lay between them. Sometimes only part, sometimes all, of the cochlea (round window to apex) was included. In many DC experiments acoustic stimulation was continued during the polarization and the responses were observed continuously on the oscilloscope. After the first few experiments the recording amplifier was provided with smaller inter-stage coupling condensers than usual to avoid artifacts from "blocking" by the polarizing current. In all cases the thresholds of the response were measured immediately, and again later, after treatment. The temporal bones were fixed and later studied histologically.‡ The histological changes observed in the serial sections were summarized in charts such as appear in Fig. 2, 3 and 4.

Results. When care was taken to avoid

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‡ The histological preparations were made by Miss A. M. Denk under the direction of Dr. W. P. Covell. We are greatly indebted to Dr. Covell, both for his helpful advice and for the free use of his laboratory facilities in McMillan Hospital (Washington University).

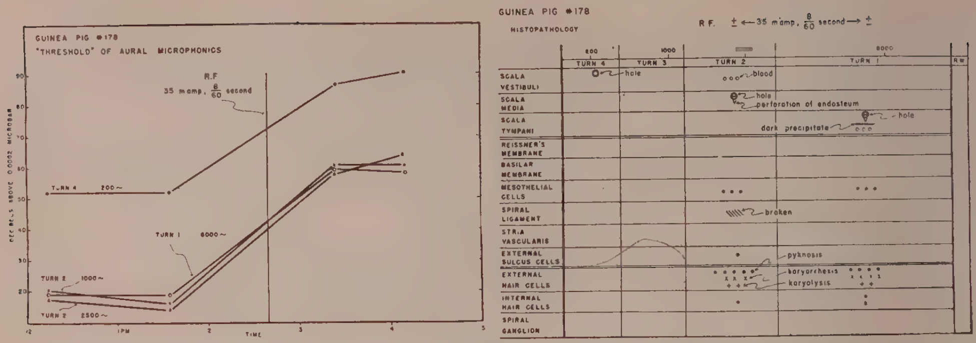

FIG. 4.

Effects of RF; 35 milliamperes for about 0.13 sec. Mild local injury at Turns 1 and 2, but great rise in thresholds for all frequencies tested. This is the typical effect of the formation of a bubble within the cochlea, either from electrolysis or heating.

polarization at the electrodes and to keep the current flow steady and below about 40 microamperes reversible modifications of the electrical responses of the cochlea were observed(3). With stronger DC currents (up to about 100 microamperes) the intra-aural muscles and sometimes other muscles of the head and neck were stimulated. When this occurred the cochlear responses were temporarily obscured by muscle action potentials. Changes in transmission occurred, particularly for low tones, and were reflected in corresponding changes in amplitude and shape of the patterns on the oscilloscope(5).

In addition, following DC current flow of 40 to 100 microamperes for 30 seconds or more, permanent elevations in the thresholds of response were produced (Fig. 2). Most of these changes were clearly correlated, according to the place principle(2), with signs of local necrosis found on microscopic examination. For example, necrosis at the apex caused profound and fairly selective depression of the cochlear microphonics and action potentials in response to a 200 cps tone. The necrosis was apparently caused chiefly by heating. It was confined to the immediate neighborhood of the tips of the wires and was very marked in cases in which the hole did *not* quite penetrate through the bone to the stria vascularis. The cells of the organ of Corti (and others) showed pyknosis, karyolysis and karyorrhexis and a loss of staining reactions and cellular structure generally. The holes in the bone often contained a black deposit (probably carbon) that tested negative for iron and for silver and was insoluble in concentrated sulphuric acid.

Sometimes, however, a large rise in the threshold of response for low tones (measured at the apex) occurred although DC current was applied only to the basal and/or second turns and although the organ of Corti was later found normal. Likewise four trials with very brief (less than 1 second) but strong (35 milliamperes) RF currents (4.0 megacycles) caused more widespread impairment of response than can be accounted for by the pathological changes. (See Fig. 4).

The explanation of many of the changes of threshold for low tones was found to be the formation of bubbles of gas inside the cochlea. (Fig. 5). DC currents of 100 microamperes passed between 2 of our silver wires immersed in boiled isotonic salt solution produce visible bubbles unless the cathode has previously been coated electrolytically with silver chloride. The bubbles are due to electrolysis and appear even when the solution has previously been boiled to drive off dissolved air. AC and RF currents also form bubbles in the cochlea, although much more current is required, probably chiefly from heating and thus driving out

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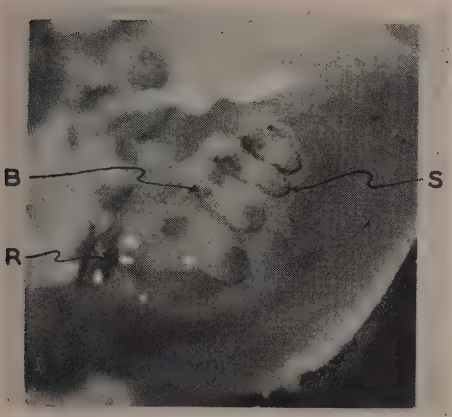

Fig. 5.
The bone of the cochlea has been dissected off under saline solution, leaving the membranous labyrinth nearly intact. The pigmented stria vascularis (S) is clearly visible in Turns 2, 3, and 4. A bubble (B) is still present in scala vestibuli of Turn 2. A larger bubble escaped during the dissection. The round window (R) is at the lower left. In the first turn the stria vascularis, spiral ligament and basilar membrane have been removed, exposing the spiral lamina. White spots are reflections from the surface of the saline solution. Treatment: DC; 100 microamperes for 1 minute.

dissolved gases.

We have noticed incidentally two anatomical features of the guinea pig's cochlea that may be of importance to electrophysiologists, as follows:

1. In the first turn the spiral ligament is separated from the scala tympani by a thin spiral shelf of bone. This shelf appears like a deposit of bone in the surface layers of the dense, non-vascular tissue that covers the tympanic half of the spiral ligament. A hole drilled into the spiral ligament behind this shelf is likely to bring an electrode into more direct contact with scala media than with scala tympani.

2. Within the modiolus a bony partition separates the channel that contains the spiral ganglion from the hollow central core that contains the cochlear nerve. Nerve fibers and blood vessels penetrate this partition freely but the holes are so small that it forms an effective barrier against any micro-electrode that may be thrust outward from the cranial cavity through the internal auditory meatus.

Summary. Direct, alternating and radiofrequency currents of 40 microamperes or more were passed between silver wire electrodes introduced in various positions within the guinea pig's cochlea. Irreversible depressions of electrical responses, both microphonic and action potentials were produced. Microscopic examination showed that two important mechanisms were a) necrosis due to heating and b) formation of bubbles by electrolysis or by heating. Reversible physiological effects can also be produced by weaker DC polarization.

Two details of the bony anatomy that may be of electrophysiological significance are described.

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Effects of Progesterone upon Ovulation in the Rhesus Monkey.* (18230)

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The hormonal control of ovulation in the primate is not very well understood, and it is extremely difficult to obtain ovulation consistently in the monkey by experimental means. There are a few authentic cases reported where ovulation has been produced experimentally with gonadotrophins(1-5).

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* This investigation was aided by grants from The Anna Fuller Fund, the National Cancer Institute (U.S.P.H.S.), and the James Hudson Brown Fund of Yale University School of Medicine.

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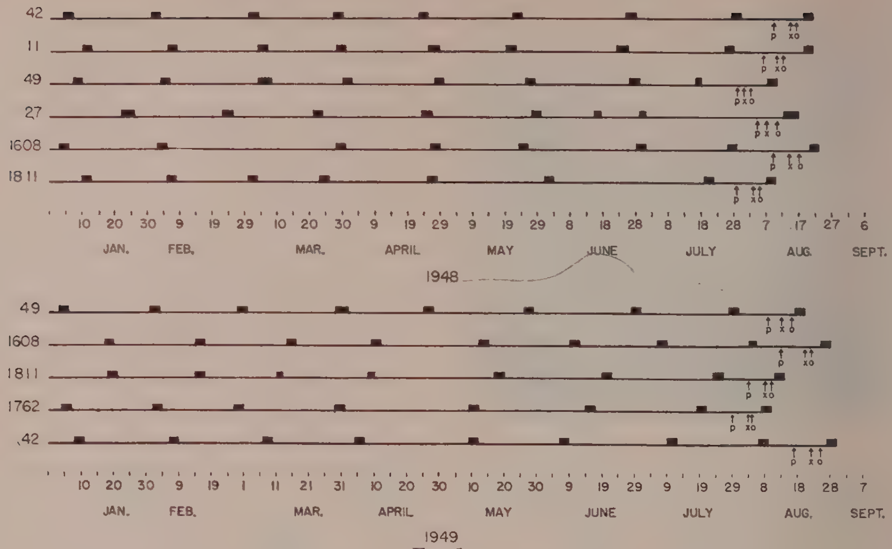

FIG. 1.

The menstrual cycles for each monkey used in the experiments are plotted from January 1 to the end of the experiments. The black blocks represent the periods of bleeding, P the time progesterone injections were begun, *X* the time injections were stopped, and 0 the time laparotomy was performed.

However, it seems probable that most attempts have resulted in failure. From the published work and personal experimentation it seems quite evident that ovulation is due to a relatively sensitive balance of gonadotrophic hormones which is somewhat difficult to reproduce by experimental means. The fact that progesterone plays such a definite rôle in the mechanism of ovulation in the rat(6), and fowl(7), together with the increasing evidence that in mammals progesterone is always produced in the maturing follicle before ovulation, suggests that this hormone might play some rôle in the mechanism of ovulation in the primate. In most colonies the female monkey runs anovulatory cycles during the summer months, which makes this animal the most favorable primate for experimentation on ovulation. It is the purpose of this paper to report the effects of exogenous progesterone administered during the middle trimester of the menstrual cycle during the summer anovulatory period.

Material and methods. The menstrual cycles of the normal female monkeys in our colony were followed routinely. Each of 6 adult female monkeys received 0.5 mg of progesterone daily for 3 to 6 days beginning on the 10th to 14th day of the menstrual cycle during August of 1948. The day that the injections were begun depended somewhat on the average length of the cycles, which are plotted in Fig. 1. The progesterone was dissolved in sesame oil so that the injected dose was contained in 0.2 cc of oil. The month of August was chosen as the period when there would be the least chance of ovulation or of the presence of a former corpus luteum that could be confused with a recently formed one. Each animal was laparotomized 2 to 3 days after the last injection of progesterone, and both ovaries were carefully examined for the stage of development and possible ovulation. The experiment was repeated in August of 1949 with the modification that the progesterone injections were started on the 10th

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TABLE I. Effect of Exogenous Progesterone Upon Ovulation in the Monkey. Each animal received 0.5 mg daily.

Animal No.	Day of cycle started	Days treated	Laparotomy Day of cycle	Laparotomy Day after last inj.	Ovulation	Remarks
		A. During anovulatory summer period of 1948				
42	13	6	21	2	—	Preovulatory follicle
11	12	5	19	2	—	Extensive adhesions
49	12	3	17	2	—	Preovulatory follicle
27	12	4	18	2	—	Large follicle
1608	14	5	22	3	+	Corpus luteum well formed
1811	10	5	17	2	—	Two large follicles
		B. During anovulatory summer period of 1949				
49	11	5	18	2	—	No large follicles
1608	10	6	19	3	+	4 day corpus luteum
1811	11	5	18	2	+	2 day ” ”
1762	11	5	17	1	+	Just ovulated
42	11	5	19	3	—	No large follicles

or 11th day of the cycle and were all continued for 5 to 6 days, with the idea that this would tend to insure the presence of the progesterone at the right stage in the growth of the follicle. Some of the newly formed corpora lutea were excised from the ovary, fixed in Bouin's fluid, imbedded in paraffin, sectioned at 7 μ and stained with Harris' hematoxylin and triosin.

Results. For the most part a single follicle develops in the ovaries of the monkeys in this colony and proceeds to approximately the preovulatory stage, but ovulation does not occur during the summer anovulatory period of June to September. There are, nevertheless, cases where more than one follicle enlarges or where no enlarged follicle can be found. The enlarged follicle gradually undergoes atresia and the menses appear at approximately the same time as if ovulation had occurred. It will be seen from Fig. 1 that there may be a somewhat greater variation in the length of the menstrual cycle during this period.

The results of the experiments are summarized in Table I. Ovulation was not readily induced in the experiment performed during the summer of 1948. It occurred in only one of the 6 monkeys tested. One other animal had such extensive adhesions around the ovaries that it was impossible to determine their development. Two others had what were diagnosed grossly as normal preovulatory follicles. Another had a single follicle which had the pale appearance often seen in cystic follicles, and the sixth animal had 2 large follicles corresponding to preovulatory follicles in size and appearance.

In repeating the experiment during the summer of 1949, 4 of the monkeys used in 1948 and one additional monkey were tested. Under the experimental regime in effect during this period 3 of the animals ovulated (Table I). The other two animals did not have preovulatory follicles. The age of the corpora lutea found in animals No. 1608 and No. 1811 was determined on the basis of the histological descriptions of Corner, Hartman and Bartelmez(8).

In both experiments menstruation followed regularly 3 to 8 days after the last injection of progesterone whether ovulation had occurred or not (Fig. 1). There appeared to be very little correlation between the number of days of treatment and the length of the period before bleeding. However, in the second experiment, where all of the animals were injected the longer period of time (5 to 6 days), the average interval was only 4 days, as compared to 5.5 days for the other group.

Discussion. It is realized that some variation exists among colonies as to whether ovulation occurs normally during the summer months. In our colony, however, the author

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has seen no spontaneous ovulation between June and September, although he has performed at least 50 laparotomies during these months. This fact agrees with the published findings of Hartman(9). While the results reported here are presented with the full realization that they are not conclusive, the author feels that they do compare very favorably with the better results of experimentation on ovulation in the monkey by other means. The single ovulation in the summer of 1948 might possibly be considered a chance occurrence. However, it is felt that the number of ovulations in 1949 were too frequent to be due to chance.

Accepting the above results to mean that progesterone produces ovulation in the monkey during the anovulatory summer period, provided a follicle of sufficient size is present, the monkey can be added to such other species as the rat(6) and the chicken(7), in which progesterone causes ovulation. It is also probable that the ovulation is the result of the release of LH from the hypophysis intermediated by the progesterone(10,11). This interpretation fits very well with the finding that progesterone is produced by the preovulatory follicle in all mammals in which it has been tested. The presence of progesterone has also been detected in the blood of the monkey(12) and of women(13) before ovulation.

It is interesting that the next bleeding seemed to occur in relation to the last injection of progesterone and not to the presence or absence of a corpus luteum. The apparent lack of correlation between the length of progesterone treatment and the number of days before menstruation(14) is probably not significant since the material was not adequately controlled for this factor.

Summary. Ovulation occurred in 4 of 11 adult female monkeys which had received 0.5 mg of progesterone daily for 3 to 6 days beginning on the 10th to the 14th day of the menstrual cycle during the anovulatory summer period. Menstruation began from 3 to 8 days after the cessation of the progesterone treatment whether ovulation occurred or not.

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Free Glutamic Acid Content of Viridans Streptococci and Some Trained Resistant Strains.* (18231)

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Gale(1) and Taylor(2) have determined the internal glutamic acid content of many different species of bacteria. Gale(3) has shown that sulfathiazole interferes with the condensation of glutamic acid into bacterial protein. Since the assimilation from the medium into the cell was not blocked, it might be expected that glutamic acid would accumulate in the cell in the presence of sulfathiazole. Gale found this to be true. He also reported(4) that resistance to sulfa-

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* Aided by a grant from The Council on Research and Creative Activity of the University of Colorado.

† Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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thiazole in *Staphylococcus aureus* appeared to be accompanied by a large amount of free glutamic acid. As evidence to support this statement he compared the values found for two strains of susceptible and two strains of naturally-resistant *S. aureus*. In previous investigations of the properties of viridans streptococci, we have trained strains of this group to resistance to sulfathiazole(5). Later, strains were also trained to increased resistance to penicillin, aureomycin,‡ and streptomycin. The penicillin and aureomycin-resistant strains were highly resistant to sulfathiazole, but the streptomycin-resistant strains were usually only slightly changed in their susceptibility to sulfathiazole(6). This paper reports the findings of an investigation of the free glutamic acid content of these resistant variants and the parent strains. The results would appear to have added interest in view of the fact that it has recently been found in our laboratory that trained sulfathiazole-resistant variants require pteroylglutamic acid, while the parent strains do not(7).

Experimental. Nine strains of viridans streptococci, 7 strains trained to sulfathiazole resistance, 6 to aureomycin, 5 to penicillin, and 6 to streptomycin resistance, and 2 enterococci were used for the determinations of glutamic acid content. One enterococcus was obtained from the American Type Culture Collection. The other enterococcus and the 9 viridans streptococci were obtained from nose and throat cultures, urine, and pus. They were classified by biochemical tests. The resistant strains were derived from the viridans group by culturing in increasingly higher concentrations of the inhibiting agents in broth. The inhibiting concentrations for all organisms were determined by inoculating 5 fold dilutions of the agents in tryptose phosphate broth, with 1 drop of a 1-1,000 dilution of a 24-hour broth culture. Differences in turbidity of broth cultures of parent and resistant strains were equalized before diluting. All tests were repeated at least 3 times. The inhibiting concentration was the lowest concentration completely preventing growth. The free glutamic acid content of the cells was determined by the method developed by Gale(8). This method depends upon the manometric measurement of CO_2 liberated from the amino acid by the specific decarboxylase for the acid. A culture of *Clostridium welchii*, SR 12, served as the source of the decarboxylase. Each organism, the glutamic acid content of which was to be determined, was grown in 300 ml of tryptose phosphate broth with .1% yeast extract, and harvested at various times, washed twice with distilled water, and finally suspended in about 4 ml of distilled water. This suspension was divided into 2 parts. One part was used to determine any free glutamic acid carried down with the cells from the medium. The other was placed in boiling water for 10′ to release the glutamic acid inside the cell. One ml of cell suspension and 1.2 ml of M/5 acetate buffer pH 4.5 were measured into the main compartment of a Warburg flask and 1 ml of the decarboxylase preparation was placed in the side arm. The CO_2 evolved from the boiled cells less that from the control was expressed in microliters of glutamic acid, as Gale(8) has done for his values. (The values may be changed to micromoles of glutamic acid by dividing by 22.4.) The weight of the cells used was determined by drying an aliquot of the suspension and weighing.

Results and discussion. Table I shows the increase in resistance attained by growing the parent strains in increasing concentrations of sulfathiazole, aureomycin, penicillin, and streptomycin. The increase in resistance to sulfathiazole found in the aureomycin, penicillin, and streptomycin-resistant strains is also given in order to point out the correlation between the internal glutamic acid content and the resistance to sulfathiazole. All

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TABLE I. Increase of Resistance in Trained Strains to Sulfathiazole, Aureomycin, Penicillin, and Streptomycin and the Concomitant Increase in Resistance to Sulfathiazole.

	Resistant variants of						
	Sulfathiazole	Aureomycin		Penicillin		Streptomycin	
	Fold increase to	Fold increase to		Fold increase to		Fold increase to	
Culture No.	Sulfa	Aureo	Sulfa	Pen.	Sulfa	Strepto.	Sulfa
1	300	100	300	400	200	200	5
4	300	100	300	1000	300	200	5
6	300	300	300	400	300	200	5
17	300	2000	300	1000	300	5000	5
23	300					1000	5
27	30	100	30				
57	30	100	300	1000	300	10,000	30

TABLE II. Free Glutamic Acid Content of Parent and Resistant Cells.

	Culture No.	Hr of growth at 37°C					
		6	8	10-12	16-18	24	36
		µl/100 mg cells					
Viridans	1		0	0	0	0	
	4	0	0	0	0	0	
	6		0	0	0	0	
	17			0	0	0	
	23					0	
	27					0	
	57					0	
	18		0	0		0	
	46		0	0	0	0	
S. fecalis	9790	163	246	205	287	263	
	47	156	260	162			
Sulfathiazole—r.v.*	1	168	220	145	120	105	
	4	142	128	181	92		
	6	226	311	203	118	68	
	17	134		168	114	284	
	23		118	180	113		
	27		170	201	135		
	57	284	399	353	162		
Aureomycin—r.v.*	1		347	351	270		
	4		208	234	80		
	6		258	262	235		
	17	158		187	174		
	57	179	333	234	139		
Penicillin—r.v.*	1		129	175	107		
	4		372	378	360		
	6		301	234	161		
	17	290		204	140		
	57		308	328	183		
Streptomycin—r.v.*	1				0	0	0
	4				0	0	0
	6				0	0	0
	17				0	0	0
	23				0	0	0
	57		388	309	147		

* Resistant variant.

of the strains grown in sulfathiazole, aureomycin, and penicillin have increased 30-fold or more in their resistance to sulfathiazole. Only one of the 6 strains trained to resistance to streptomycin showed a 30-fold increase to sulfathiazole. This was the strain which increased 10,000-fold in resistance to streptomycin. The other strains showed only a slight increase in sulfathiazole resistance.

Table II records the microliters of glutamic acid per 100 mg of dry cells found in the parent and resistant strains, and in 2 strains of *Streptococcus fecalis*. Preliminary determinations had shown that the greatest amount of glutamic acid was found just at the time the cells had reached the end of active growth. This point was determined by following the increase in turbidity of the broth culture with the Klett-Summerson photoelectric colorimeter. In order that the time would not be missed when the greatest amount of glutamic acid would be present, several determinations were made at various times before and after the end of active growth. Each value given is for a single determination. Repeated determinations made on several of the organisms gave some variation in values at a given time of harvesting, but those showing positive values were always positive, and those showing negative values were never found to be positive. It is interesting that, under the conditions of this experiment, no free glutamic acid was found in any of the gram positive viridans streptococci. Taylor (2) reported very low values for some, but no completely negative values for any gram positive organism. His manometric determinations were made at 30°C, which Gale(7) reports as optimum for decarboxylase activity. Our determinations were made at 37°C. The values found for our known strain of *S. fecalis* were comparable to those found by Gale(1) for *S. fecalis*.

Both strains of *S. fecalis* and all of the 8 strains trained to sulfathiazole resistance by growing in sulfathiazole showed a high internal concentration of free glutamic acid. All of the trained aureomycin and penicillin-resistant strains also showed a high internal concentration of glutamic acid, but only the one streptomycin-resistant strain which had a concomitant 30-fold increase to sulfathiazole showed the presence of free glutamic acid. The other streptomycin-resistant variants, whose sulfathiazole resistance increased only 5-fold, gave negative results when determinations for glutamic acid were made. High values for internal glutamic acid apparently accompany resistance to sulfathiazole, whether this is natural resistance, resistance induced by growing in sulfathiazole, or resistance accompanying an increase of resistance to other agents.

Other studies in our laboratory, to be reported later(6) have shown that the same biochemical properties are seen in trained sulfathiazole, aureomycin, and penicillin-resistant strains, but these properties are often not found in trained streptomycin-resistant strains. These are properties characteristic of enterococci. In our studies, glutamic acid in concentrations similar to those found in *S. fecalis*, were found in the resistant cells.

Gale(4) has made the suggestion that the large amount of glutamic acid in resistant cells may itself be responsible for the increased resistance to sulfathiazole, since he found that in media containing graded amounts of glutamic acid, the media with the most glutamic acid required the most sulfathiazole to prevent growth of *S. aureus*. It may be, however, that the resistant cells become so because of a change in metabolism which eliminates the necessity of using the free glutamic acid taken up by the cell from the external environment, and so allows it to accumulate in the cell. Since it has previously been shown(7) that trained sulfathiazole-resistant strains of *Streptococcus mitis* require pteroylglutamic acid (PGA), while the parent susceptible strains do not, one possible explanation for part of the increase in glutamic acid might be that the resistant cells no longer are able to use free glutamic acid for the synthesis of PGA. The amount needed for this synthesis would hardly be enough to account for the differences noted here between susceptible and resistant cells, however. Some other products needed by the cell, and synthesized from free glutamic acid in the parent susceptible strains, may be supplied from another source in the resistant cell and allow more accumulation of the glutamic acid. Further information on this subject might be obtained by employing Gale's technics for preventing internal metabolism, and measuring the effect on the glutamic acid content of the susceptible cells, since at present we do not know whether these cells use glutamic acid from the environment or synthesize it themselves.

Summary. Free glutamic acid was found in all of the trained sulfathiazole, aureomycin, and penicillin-resistant strains of viridans streptococci tested, but not in the parent strains. The aureomycin and penicillin-resistant strains had also increased in sulfathiazole resistance. No glutamic acid was present in 5 trained streptomycin-resistant strains which had not increased appreciably in sulfathiazole resistance, but was found in one strain which had increased in sulfathiazole resistance. Glutamic acid appears to accompany sulfathiazole resistance regardless of how this resistance is acquired. The possible relation of the free glutamic acid to the increased resistance to sulfathiazole is discussed.

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Levels and Intracellular Distribution of Coenzyme A and Pantothenic Acid in Rat Liver and Tumors.* (18232)

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The relative inability of tumor homogenates to oxidize oxalacetic acid(1,2) and the participation of coenzyme A (CoA) in the enzymatic condensation of oxalacetate and C_2 units to citrate(3,4) suggest that tumors might be relatively deficient in this coenzyme. Earlier work pointed to a low level of pantothenic acid (PA) in tumors(5), but the methods available at that time for liberating the vitamin from bound forms were probably inadequate(6). Therefore the contents of coenzyme A and pantothenic acid were determined in several types of transplantable rat tumors. Since the enzymes involved in the Krebs tricarboxylic acid cycle appear to be relatively concentrated in the mitochondria of normal liver(7) a comparison was also made of the intracellular distribution of these factors in normal rat liver with that in azo dye-induced rat liver tumors.

Experimental. The transplantable tumors were collected from multiply-inoculated rats[†] bearing the Walker carcinosarcoma, the Jensen sarcoma, or the Flexner-Jobling carcinoma. Only firm tumors less than 1 cm in diameter were analyzed. The minced tumors were homogenized in ice-cold isotonic saline in the Potter-Elvehjem apparatus to give a final tissue concentration of 10%. The liver tumors were taken from rats fed for 5 months on a low riboflavin, semi-synthetic diet(8, diet 3) containing 0.06% 4-dimethylaminoazobenzene. The liver tumors were perfused *in situ* with ice-cold isotonic saline by injection into the aorta at the thoracic level; the aorta below the coeliac artery was clamped off and the fluid allowed to escape by severing the vena cava above the liver. This perfusion route was necessary since liver tumors, unlike liver, are supplied only by arterial blood

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(9). Firm tumors 1 cm or less in diameter were carefully dissected from the livers, pooled, and washed in ice-cold saline. All of the normal livers were perfused with saline via the vena cava as described by Price *et al.*(10). The normal rats (160-200 g) and the rats (120-150 g) bearing the transplantable tumors were maintained on a grain diet (11). The preparation and differential centrifugation of the liver and liver tumor homogenates were carried out in 0.88 M sucrose essentially as previously described(10, 12). The nuclei, mitochondria, and microsome fractions were collected at 600 g for 10 minutes with two washings, 18,000 g for 10 minutes with one washing, and 120,000 g for 36 minutes without washing, respectively. The tube and pellet containing the latter fraction were rinsed with the sucrose medium and the washings added to the supernatant fraction. A refrigerated International centrifuge with a high-speed attachment was used to separate the first two fractions; the microsome fraction was sedimented in an air-driven ultracentrifuge.

All of the homogenates and 10% suspensions of the intracellular fractions in the sucrose medium were heated for 10 minutes in a boiling water bath to preserve the bound forms of pantothenic acid and suitable dilutions were made with distilled water for analysis. CoA was determined by the sulfanilamide acetylation method of Kaplan and Lipmann(13). Total PA was determined microbiologically with *L. arabinosus*(14) after enzymatic digestion(6). Free PA values were obtained by applying the same method to undigested aliquots. Duplicate PA and CoA assays were conducted on each sample and agreed within 15% for the more concentrated samples. With some of the lower potency tumor fractions, a 30-50% variation occurred and the average of the two values is reported unless additional determinations were made.

TABLE I. Coenzyme A and Pantothenic Acid Content of Certain Transplantable and Induced Tumors in the Rat.

Tumor	Sample No.	Coenzyme A*	Pantothenic acid†	
			Total	Free
Walker carcinosarcoma	1	9.8	5.7	.5
	2	12.0	15.3	.1
	3	7.2	5.3	1.9
Jensen sarcoma	1	11.2	4.5	.0
	2	8.4	6.8	.4
	3	8.4	7.1	.0
Flexner-Jobling carcinoma	1	11.9	12.3	.0
	2	16.8	19.7	.2
	3	7.6	7.0	.0
Induced liver tumor	1	18.8	15.2	.6
	2	28.8	22.2	1.1

* Lipmann units per g fresh tissue.

† μg per g fresh tissue.

Results and discussion. From the data in Table I it appears that the transplantable tumors contained roughly similar amounts of coenzyme A and pantothenic acid, although fairly wide variations were observed with some of the samples. The induced liver tumors (Tables I and II) on the other hand contained appreciably more of these factors than the transplantable tumors. In comparison with several normal rat tissues analyzed by similar methods, however, all of these tumors contain only about 1/2 to 1/10 as much of these factors(13, and Table II). The PA values in Table I are about 20 to 30% higher than those previously reported for similar rat tumors (5). This is undoubtedly a result of a more complete release of PA from bound forms(6). The low amount of coenzyme A in these tumors thus parallels the finding of Potter *et al.*(1,2) that homogenates of these tissues contain low amounts of the enzymes that oxidize oxalacetic acid.

The intracellular distribution of CoA and PA in normal rat liver and in azo dye-induced rat liver tumors is given in Table II. In gen-

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TABLE II. Intracellular Distribution of Coenzyme A and Pantothenic Acid in Normal Rat Liver and Induced Rat Liver Tumor.

	Coenzyme A*				Pantothenic acid†							
					Total				Free			
	Liver		Tumor		Liver		Tumor		Liver		Tumor	
Sample No.	1	2	3	4	1	2	3	4	1	2	3	4
Fraction												
Whole homogenate	174	166	30	15	126	118	17	14	—	1.9	4.8	5.2
Nuclei	39	40	4.2	2.9	33	24	3.0	2.9	—	.2	1.4	.8
Mitochondria	95	82	9.4	4.4	63	42	2.6	1.8	—	.3	1.0	.8
Microsomes	3.8	8.6	0.0	0.3	5.3	5.9	.4	1.2	—	.1	.4	.0
Supernatant	33	57	12	6.3	30	32	5.5	5.7	—	.5	2.1	.9
Recovery	171	188	26	14	131	104	12	12	—	1.1	4.9	2.5

* Lipmann units per g fresh tissue.
† μg per g fresh tissue.

eral, the distribution of these two components paralleled one another closely. As expected from previous work on the distribution of the enzymes involved in the Krebs tricarboxylic acid cycle(7) a large share of the CoA was found in the mitochondrial fraction of each tissue. In the normal livers this fraction contained about half of the total amounts of the CoA and PA present. The remaining amounts of these substances were divided almost equally between the supernatant and nuclear fractions. As in the case of vitamin B_6(15) the microsomes contained little of these factors; it is quite possible that the small amounts found were due to contamination by supernatant fluid since the microsomes were not washed by resuspension. The tumors, on the other hand, not only contained much less CoA and PA but the distribution of these factors among the morphological constituents appeared to differ from that of normal liver. Thus the supernatant fraction accounted for nearly half of the vitamin and coenzyme present in the tumor and the mitochondria and nuclei contained the remaining amounts in the order named. As in the normal liver only very low amounts were found in the tumor microsome fractions.

While few conclusions can be drawn from the free PA values given in Table II, it would appear that there was a much higher content of free pantothenic acid in the liver tumor than in the normal liver. However, the data in Table I indicate that when these liver tumors are analyzed quickly after death of the animal the content of free PA is considerably less although it is still higher on a percentage basis than in normal liver. This discrepancy is probably due to the fact that the fractionation procedure required several hours to complete. Even though the homogenates and fractions were held at 3 to 5° throughout this time it is quite probable that some enzymatic liberation of PA occurred. Similarly, the low recoveries of free PA shown in Table II may have been due to a greater liberation of PA in the whole homogenate than in any of the isolated fractions.

The recoveries of CoA and total PA in the fractionations were reasonably good (89 to 113%) in the case of normal liver but they tended to be low (71 to 93%) with the liver tumor samples. The reason for this difficulty is not known.

Summary. The intracellular distribution of pantothenic acid and of coenzyme A have been determined in normal rat liver and in azo dye-induced rat liver tumors. These substances were also determined in three kinds of transplantable rat tumors. All of the tumors were very much lower in these components than normal liver. The majority of each factor in the liver was contained in the mitochondria; however, in the liver tumor the supernatant fraction contained the largest amounts. The remaining amounts were distributed between the nuclear and supernatant fractions of the liver and the mitochondrial

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and nuclear fractions of the liver tumor. The microsomes from each tissue contained only small amounts of the vitamin and coenzyme. The finding that these tumors contain low levels of coenzyme A is in harmony with the observation of Potter and his associates that homogenates of such tumors contain low amounts of the enzymes that oxidize oxalacetic acid.

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A Complement Fixation Test for Equine Virus Abortion.* (18233)

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(Introduced by Roy C. Avery)

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Equine virus abortion (E.V.A.) was first described as a clinical entity, of unknown etiology, by Dimock and Edwards(1). Subsequent reports(2-8) established the viral etiology, and more fully characterized this disease. E.V.A. is an epizootic contagious disease of Equidae characterized by abortion, usually in the late stages of pregnancy, without signs of infection of the mare before or after parturition. The most striking pathological lesion noted in the aborted fetus are small foci of necrosis in the liver. Other changes worthy of note are hemorrhages in the abdominal and thoracic organs, edema and congestion of the lungs, and pleural effusion. Necrosis and intranuclear inclusions are the chief histopathological features. E.V.A. has been reported(6,9) from 12 states in this country, and five foreign countries. Although data regarding the incidence of this disease in the United States are incomplete, the majority of cases appear to occur in central Kentucky, principally on thoroughbred farms. abortion rates ranging from 10% to 85% in exposed groups of animals indicate the extreme degree of contagiousness of the virus. Little, however, is known concerning the mode of transmission of E.V.A.; apparently it is not venereal, since the stallion has not as yet been implicated. The disease has been induced artificially by feeding pregnant mares unfiltered infected placenta or fetal tissue, and by the injection of bacteria-free filtrates of infected tissue. The incubation period of such infected animals is from 18-30 days.

The host range of the virus appears to be narrowly limited. Small success has attended the efforts of those who have attempted to grow this virus in animals other than the natural host. Dimock, Edwards, and Bruner (6) were unable to demonstrate infection in mice, rabbits, guinea pigs and chick embryos. Serial passage through guinea pig foetuses(10) has failed to maintain the virus. Histopathological evidence of infection in newborn Syrian hamsters has been adduced by Goodpasture and Anderson(11,12). Other investigators (9,10) have not succeeded in demonstrating

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infection in day-old hamsters. There is therefore no method by which the presence of the virus of E.V.A. can be unequivocally demonstrated save by the injection of the presumptively infected material into a pregnant mare, with resulting abortion of a foal showing the characteristic gross and microscopic pathological changes. Such a test is impracticable, save for a very few laboratories. (It may be pointed out, in passing, that injection of pregnant mares with infected fetal tissue is by no means followed by abortion in all cases.) A simple, convenient, rapid and unequivocal test for detecting the presence of this virus would be highly desirable. From the standpoint of the rather considerable economic loss sustained by breeders of thoroughbred horses, a test which would give information on the presence of antibodies might be of great value in the diagnosis and prognosis of E.V.A., and help in evaluating the efficacy of prophylactic vaccines. A specific serological test should meet all these requirements. Since the complement-fixation test has been successfully used in other virus infections, it seemed preferable to attempt to devise such a test for E.V.A. This paper constitutes a report on the complement-fixation test developed in these laboratories for the detection and measurement of the specific antigen and antibody of E.V.A.

Methods and results. Preparation of antigens. Since the lungs and liver, and to a lesser extent the spleen, of aborted foals, have been found to be the most heavily infected organs, these organs were used as potential sources of antigen. The frozen tissue (which had been preserved at –18°C for from 2 to 9 months) was cut into blocks averaging about 1 cm on a side, and the blocks washed in repeated changes of ice cold physiological saline until no further trace of hemoglobin could be obtained. After overnight storage at 4°C in saline, the washed tissue blocks were drained, blotted nearly free of moisture, weighed, refrozen at –18°C, ground to a smooth paste, and diluted 1:2 with cold saline on the basis of the weight before refreezing. Low-speed centrifugation (3000 r.p.m. for 10 minutes at 4°C) was found, in early experiments, to yield an antigen which appeared to be unsatisfactory for complement-fixation tests. The ground tissues were therefore centrifuged at 13,500 r.p.m. for 30 minutes at 4°C, and a crystal-clear hemoglobin-tinged supernatant obtained. This supernatant, without further treatment, was tubed in convenient small amounts, and stored at –18°C until used. Organs from normal, uninfected animals were treated in the same manner, for use as one control of antigen specificity in the complement-fixation test.

Preparation of sera. All blood specimens used in the tests were drawn with the usual aseptic precautions, allowed to clot; the expressed serum pipetted off, clarified by centrifugation, and stored in small ampoules at –18°C until use. Potentially positive sera were available for testing from horses which had acquired E.V.A. naturally, and from horses in which this disease had been artificially induced. An attempt was also made to obtain positive serum by artificial immunization of two male horses. The first horse, a yearling stallion, was given an injection into each testicle of 3 ml of a 1:2 dilution of equal parts of ground lung, liver and spleen antigen. (It may be noted here that when the testicles were removed ten days after the injection, no specific lesions could be demonstrated microscopically.) The second horse, a 2-year-old gelding, was given a single intravenous injection of 15 ml of a mixture composed of the supernatants from both low- and high-speed centrifugation of ground infected lung tissue. For serum controls, blood was obtained from normal stallions, and from normal mares with no history of E.V.A.

The complement-fixation test. Throughout all experiments we have used the 50% hemolysis end-point complement-fixation test, following the spectrophotometric method of measurement developed by Mayer *et al.*(13), and Kent *et al.*(14). A veronal-buffered sa-

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line solution(15) was used for all dilutions. In the qualitative test, a mixture of 1 p. antigen, 1 p. serum (1:5 dilution), and 1 p. complement (containing 3 H_{50} units), is allowed to incubate at 4°C for 18 hours. At the end of this period of primary incubation, 1 p. of optimally sensitized sheep erythrocytes is added to the system, followed by a second period of incubation for 45 minutes in a 37°C water-bath. Adequate controls are included in each test. Sera showing 80% hemolysis or less, and which are not anticomplementary, are considered positive, and subsequently titrated in two-fold serial dilution.

The first trial run, using as antigen the supernatant from low-speed centrifugation of a heavily infected liver, gave a negative result with serum from a proven case of naturally acquired E.V.A. Predicating the presence of antibodies in the serum, and fixation of complement by these antibodies in combination with adequate amounts of antigen, it was considered that this negative result might be a reflection of low viral content of the antigen, which had had to be diluted 1:160 to avoid non-specific fixation of complement. Accordingly, in all subsequent tests the supernatants of high-speed centrifugation of infected tissues, were employed as antigens. Four antigens were prepared by this method: liver, spleen, lung No. 1, and lung No. 2. (Each antigen was derived from a different infected fetus. Tests for non-specific fixation of complement, in the presence of 1 H_{50} unit of complement, showed that the strongest concentrations in which these antigens could be used were 1:80, 1:640, 1:40, and 1:100, respectively. These antigens, in the stated dilutions, were then set up against the serum used in the first run, and against serum obtained from the stallion which had been injected intratesticularly 10 days before bleeding. Complete fixation of the 3 H_{50} units of complement occurred with lung No. 2 antigen, with both test sera; lung No. 1 antigen showed slight but definite fixation with both sera; neither the liver nor the spleen preparations gave any fixation with either serum. None of the 4 antigens fixed complement in the presence of control serum obtained from the immunized stallion before intratesticular injection. A test to determine the optimal dilution of lung No. 2 antigen revealed strongest fixation at all dilutions of antiserum, in the highest non-anticomplementary concentration (1:100) which could be employed. Control antigen prepared from the lungs of a normal horse in precisely the same manner as the test antigen, and used in proper non-anticomplementary dilution, did not fix complement in the presence of either positive or negative sera.

Using lung No. 2 antigen it has been possible to demonstrate the presence of antibodies in the serum of seven horses with proven E.V.A., both naturally and artificially acquired, and in serum from the gelding immunized by the intravenous route. The titers of sera obtained from infected horses ranged from 1:16 to 1:320. Both of the sera obtained from the two immunized male horses showed strong fixation at 1:640. Serum from two non-immunized male horses, and from ten normal female horses with no history of E.V.A. have been consistently negative.

Conclusions and summary. The complement-fixation test described in this paper provides a means whereby the presence of antibodies to the virus of E.V.A. can be detected and quantitatively estimated in serum. The lung antigen employed in the test is specific in the sense that it does not react with sera from normal horses, and that similarly prepared antigen from the lungs of a normal horse does not react with positive sera. (Whether or not the antigen will react with antibodies to other viruses, and whether or not different strains of this virus exist, remain to be investigated.) By means of this test information may now be obtained on the immunological course of E.V.A. in diseased mares, which will perhaps help in the early diagnosis of E.V.A., and in the prognosis of this infection. The response of 2 horses to single injections of infected material has revealed the potent antigenicity of the prepara-

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tions. The possibilities of prophylactic immunizations, using the complement-fixation test to check the progress of the immunization, are obvious. Lastly, the test may be employed as a means of determining the presence or absence of the virus, whether in the natural host, experimentally infected animals, or inoculated tissue cultures. Experiments are in progress on an evaluation of the complement-fixation test, as employed for these purposes.

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A Cannula for Simultaneous Drainage of Both Cavae in Artificial Heart Experiments.* (18234)

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One of the many problems connected with a mechanical heart preparation is that of the cannulation of both the superior and inferior vena cavae. The superior vena cava may be cannulated easily through the azygous vein, which may then be ligated after removal of the cannula [Gibbon(1); Leeds, Gray, and Cook(2)]. The inferior vena cava may be cannulated through the right auricular appendage which is then ligated after removing the cannula [Gibbon(1)]. In order to simplify the cannulation, a single right angle cannula was constructed which permits blood from both the cavae to pass through it and thence flow to the pump. This procedure saves a step in the experiment, and appears to be more satisfactory than having two cannulas. It is certain to drain the entire caval flow and, therefore, should be preferable to the method of Jongbloed(3), who passes a catheter into each cava near the auricle and applies suction to draw the blood from the cavae to the pump. With the new cannula ligated in place no blood from the cavae can enter the right auricle, and it must all be diverted. The right auricle or ventricle may be opened with very little blood loss since only the coronary flow enters the operative site. The latter constitutes about 5 or 6% of the cardiac output [Harrison, Friedman and Resnick(4)].

The purpose of the present communication is to describe a right angle cannula which is passed through the azygous vein and which in one operation permits the drainage of

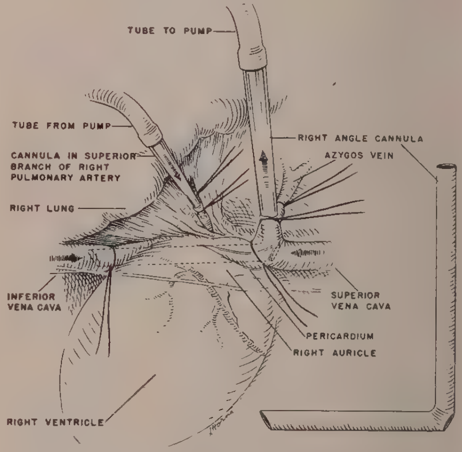

Fig. 1.

Diagram which shows cannula inserted through azygous vein into superior and inferior *vena cavae.* The ligatures prevent any caval blood from entering the right auricle. The cannula which returns the blood from the pump to a branch of the right pulmonary artery is also shown.

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blood from both the cavae. This cannula has been used satisfactorily in more than 25 experiments in dogs in which an extracorporeal shunt was established.

Description of the cannula. The cannula consists of thin-walled (26 gauge) metal tubing of uniform diameter with 2 arms at an angle of 90 degrees. The tube is open at both ends and is also open at the vertex of the angle (Fig. 1). Cannulae of 5/16 inch and 1/4 inch inside diameter were most satisfactory for the large dogs which we used. The larger cannula is used whenever possible. The shorter arm measures 8.0 cm and the end is oblique in order to facilitate insertion into the azygous vein and inferior cava. The longer arm measures 11.5 cm and the end is cut transversely for attachment to rubber tubing. The interior of the cannula is coated with silicone before it is used. *Method of insertion and removal.* The azygous vein is prepared for insertion of the cannula by dissecting it free for a distance of 2 or 3 cm from the junction with the superior vena cava. Two silk or cotton ligatures are placed around the azygous vein; the distal one is tied, and the proximal one is tied around the cannula after it is inserted. Prior to insertion of the cannula the pericardium is opened by a longitudinal incision over the superior vena cava and right auricle and a length of narrow umbilical tape is placed around the portion of the auricle which the superior vena cava joins. A second tape is placed around the inferior vena cava just inferior to the pericardial reflection. The tapes are tied after inserting the cannula through the azygous vein into the right auricle and thence into the inferior vena cava. During the passing of the cannula the operator covers the opening at the angle with the tip of his index finger to prevent the escape of blood. The tape ligatures make it impossible for any caval blood to enter the right auricle and the entire flow from both cavae is diverted through the cannula into the pump (Fig. 1).

TABLE I. Results of Survival Experiments in Which the Triple-opening Right-angle Cannula Was Used.

Exp. No.	Wt (kg)	Occlusion of cavae (min.)	Total output of pump (liters)	Avg flow (cc/min.)	Avg flow (cc/kg/min.)
1	22	31	17.8	560	25.5
2	21	13	9.6	740	36.2
3	15	26	23.2	890	59.3
4	30	25	12.1	490	16.3
5	28	28	20.6	730	26.1
6	18	32	21.7	680	37.8
7	23	46	41.5	900	39.1
8	14	41	44.8	1090	77.9
9	15	15	14.6	980	65.3
10	12	33	25.8	780	65.0
11	18	42	73.6	1750	97.2
12	18	46	71.0	1540	85.6
13	22	30	46.1	1540	70.0
14	24	21	34.0	1620	67.5
15	17	18	19.8	1100	64.7
16	15	23	16.3	710	47.3
17	29	68	72.4	1064	36.7
18	20	41	48.4	1180	59.0
19	21	34	39.8	1170	55.7
Avg	20.1	32.3	34.4	1027	54.3

To remove the cannula at the end of an experiment one cuts the tapes and the ligature around the azygous vein with a scalpel. The cannula is then withdrawn quickly and the azygous vein is ligated near its junction with the cava.

Results of experiments. In Table I are summarized the results of 19 experiments in which the entire venous flow of blood was diverted from the cavae for from 13 to 68 minutes followed by survival of the animals for an indefinite period. It is seen that flows as high as 1750 cc per minute passed through the pump. Factors which interfere with the efficiency of the mechanical heart are under study; however, the size of the cannula is undoubtedly adequate to carry the entire cardiac output with ease.

Summary. A single cannula which can be tied in place to divert the entire flow of both the superior and inferior vena cava has been described. This cannula has been useful in experiments in which an extracorporeal pump or artificial heart has been employed.

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Effect of Cortisone and Adrenocorticotrophic Hormone (ACTH) on Experimental Scurvy in the Guinea Pig.* (18235)

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In recent clinical trials of adrenocorticotrophin (ACTH) in patients with purpura hemorrhagica, it has been found that the drug regularly brings about a temporary and almost complete remission of bleeding(1). Similar effects of the hyperadrenal state were seen whether the purpura was idiopathic or associated with aplastic anemia, leukemia, or lupus erythematosus disseminatus. Indeed, one of us (C.R.)† observed disappearance of the hemorrhagic manifestations in a scorbutic patient who was treated briefly with ACTH.

As a first approach to experimental analysis of the action of these hormones in purpura, a study of the influence of induced hyperadrenalism on scurvy in the guinea pig has been made. The disease is readily produced in this species and its manifestations are well known(2-5). Furthermore, a special interest in the relation of scurvy to the adrenal has already developed from the familiar facts that the gland contains extraordinary amounts of ascorbic acid(6) and that adrenal hypertrophy is a regular accompaniment of scurvy (7-11). Schaffenburg, *et al.*(12) have recently reported that cortisone exerts a protective action in guinea pigs placed on a scorbutic diet. Their studies were undertaken on the hypothesis that certain of the mesenchymal alterations of scurvy are the result of hyposecretion of cortisone-like steroids, which results from vitamin C deficiency (13,14). Studies of the effects of ACTH were not included in this report. In the present study it is shown that the effects of cortisone on the scorbutic guinea pig are also produced by ACTH. This finding lends support to the view that deficiency of ascorbic acid does not appreciably interfere with production of adrenal corticosteroids.

Methods. The guinea pigs studied were approximately two months old and weighed 300 to 400 g. Four were given a normal Rockland vitamin C-fortified diet(15) with added lettuce while 22 received an autoclaved, vitamin C-free diet(16) in the course of 2 experiments. In the first experiment,

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TABLE I. Summary of Data.

Treatment		Duration of scorbutic diet (days)	Duration of therapy (days)	Onset of wt loss after scorbutic diet (day)	Total max. wt loss (g)	Adrenal wt (mg)	Ratio: adrenal wt (mg) / body wt (g)	Adrenal ascorbic acid (μg/100 mg)
Normal—avg of 4		0	0	—	—	145	0.46	51.3
A. None	1	20	—	8	51	—	—	0
	2	21	—	8	158	—	—	0
	3	24	—	13	50	305	1.48	0
	7	20	—	8	65	300	0.98	2.2
	8	21	—	8	75	325	0.95	0
	15	23	—	10	170	300	1.16	2.1
	15A	20	—	6	135	300	1.22	—
	16	24	—	12	165	380	1.44	—
	17	24	—	10	145	300	1.41	2.3
Avg		21.9	0	9.2	113	315	1.2	—
B. Cortisone	4	25	15	8	85	120	.52	—
	5	26	16	19	90	—	—	—
	6	34	24	21	50	160	.58	0
	9	22	35	14	40	180	.46	0
	10	24	37	14	60	190	.52	—
	14	19	10	15	55	315	.9	4.3
	18	24	15	16	90	200	.65	3.9
	19	27	18	16	65	340	1.2	0
	24	30	21	16	200	300	1.13	0
Avg		26.5	22.3	15.4	80	225	.74	—
C. ACTH	20	30	21	16	155	600	2.22	0
	21	30	20	16	195	660	2.7	0
	22	29	19	16	180	430	1.72	0
	23	30	21	23	80	520	1.58	1.5
Avg		29.8	20.3	18	150	550	2.06	—

10 scorbutic animals were studied: 5 received cortisone[‡] while 5 were not treated. Two animals received cortisone for 13 days before the scorbutic diet was started, and 3 received cortisone only after the diet had been given for 10 days. The second experiment involved 3 groups of 4 scorbutic animals. The first group was given cortisone after 10 days on the scorbutic diet. The second received ACTH,[§] again after 10 days on the same regimen. The third group was not treated. The effects of the hormones in both experiments will be examined together. Cortisone was given once daily in a dose of 12.5 mg subcutaneously. ACTH was injected every 6 hours, 6.25 mg subcutaneously. In order to demonstrate glycogen and polysaccharide complexes, the periodic acid Schiff reaction and diastase method were employed. Toluidine blue was used in order to stain acid mucopolysaccharides. The ascorbic acid content of the adrenal gland and of the blood were determined by the method of Roe and Kuether(17). The important data are summarized in Table I.

Results. 1. *Scorbutic, untreated controls.* Group I, 9 animals. Two of the animals were female and 7 male. No significant differences related to sex were noted during the study. The average duration of life was 21.9 days with extremes of 20 to 24 days. Five animals were sacrificed after their weights had fallen to about 250 g from an average of 370 g. They then appeared moribund.

[‡] The cortisone used in these studies was purchased from Merck and Co., from funds provided by the U.S. Public Health Service.

[§] The ACTH used in these studies was purchased from Armour and Co. Dosages are equivalent to Armour Standard LAIA.

17. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, v147, 399.

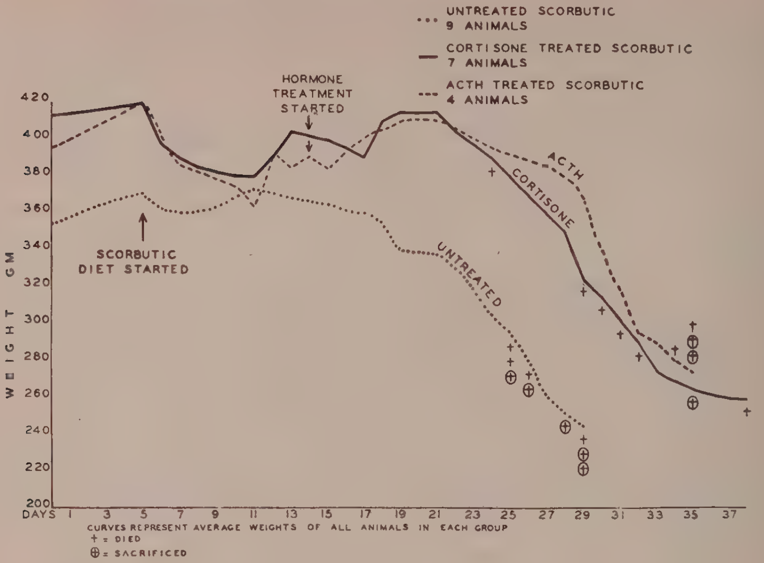


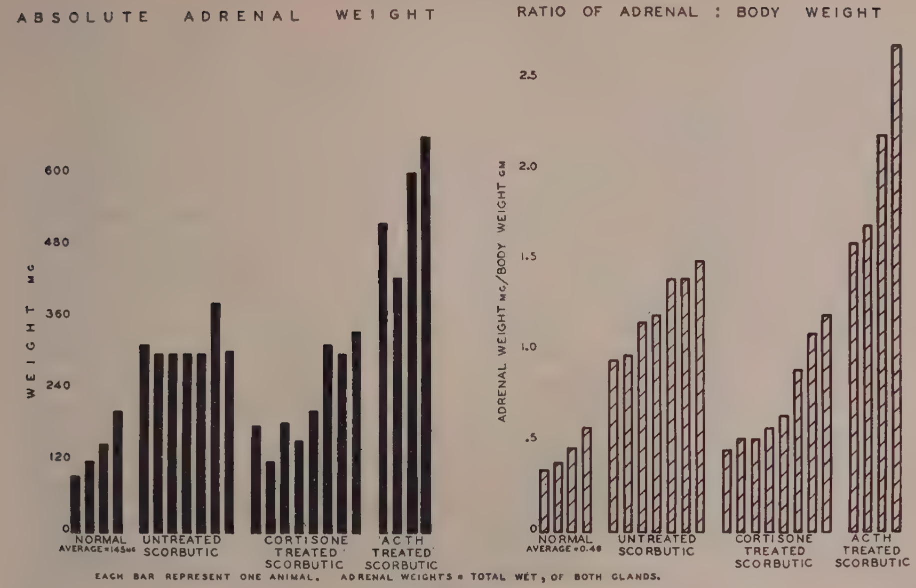
FIG. 1.

Effect of cortisone and ACTH on body weight and survival of scorbutic guinea pigs.

The clinical course was strikingly similar in all animals, resembling that described by Meyer(4). On the tenth to twelfth day, bloody diarrhea and hematuria developed in all animals, anorexia and lethargy was marked, while hind leg paralysis, tachypnea, and abdominal distention appeared in five. The weight curves in Fig. 1 reveal a failure to gain from the start of the diet, and a consistent loss of weight from about the ninth day. A very rapid fall occurred after the seventeenth day. Gross examination disclosed bladder and perivesical hemorrhage in all of the animals. Marked hemorrhage occurred in the subcutaneous tissues and muscles of the lower extremities, lungs, upper intestinal tract, cecum and spleen. There was considerable adrenal hypertrophy.

Microscopic examination of the viscera, bones and joints, muscle and subcutaneous tissue disclosed extensive, characteristic lesions of scurvy(5,10). Additional disorders present at death consisted of liver abscess in one animal and pneumonia in a second.

2. *Scorbutic, cortisone-treated.* Group II, 9 animals. Two male guinea pigs were treated with cortisone for 13 days before receiving the scorbutic diet. The hormone was then given until they died after 22 and 24 days on the diet. The course of these animals is not represented in Fig. 1. However, weight gain continued on cortisone until the scorbutic diet was instituted. The weight then levelled off for about 14 days, and finally dropped rapidly until death. In 3 male and 4 female pigs, cortisone therapy was started on the tenth day of the scorbutic diet. These animals lived for 19 to 34 days on the scorbutic diet, an average of 26.5 days. After 7 days of therapy, a relatively steep fall ensued and continued until death (Fig. 1). The onset of clinical scurvy was approximately one week later than in the scorbutic controls. In two animals death apparently resulted from inanition and pneumonia after 19 and 25 days of the diet. No gross hemorrhage was seen in either. The remaining 7 animals, however, did present hemorrhages, which seemed appreciably less than in the untreated group, although bloody diarrhea, hematuria and hind leg paralysis had appeared in most of them. One animal was sacrificed when moribund, the remainder dying without interference. Microscopic findings are reported



EACH BAR REPRESENT ONE ANIMAL. ADRENAL WEIGHTS = TOTAL WET, OF BOTH GLANDS.

Fig. 2.
Effect of cortisone and ACTH on adrenal size.

with the ACTH-treated group.

3. *Scorbutic, ACTH-treated.* Group III, 4 animals. Four female guinea pigs were started on ACTH on the tenth day of the scorbutic diet. The course of ACTH was continued for 19 to 21 days, with three animals living 30 days, and one 29 days, an average of 29.8 days. In all cases a modest weight loss after initiation of the diet was followed by a plateau or slight rise (Fig. 1), but this was followed by a progressively rapid fall until death. Two moribund animals were sacrificed. In the other two, marked pulmonary edema was found at autopsy. Bloody diarrhea, hematuria and hind leg paralysis were observed terminally in three cases, but these signs developed about eight days later than in the controls of Group I. The hemorrhagic manifestations were roughly comparable to those occurring in the cortisone-treated group, and again seemed rather less than those of the untreated scorbutic controls. Microscopic examination generally confirmed the gross impression of amelioration of some of the scorbutic manifestations in the treated animals. The untreated scorbutic animals showed marked subcutaneous, intramuscular, periosteal, and epiphyseal hemorrhages. In the ACTH- and cortisone-treated animals, these regions showed hemorrhage but it was less extensive. All of the scorbutic animals had varying degrees of degeneration of muscle with no discernible differences between the treated and untreated. The histochemical technics employed failed to disclose any abnormalities of basement membranes or of perivascular tissues. Whereas glycogen was histochemically not demonstrable in the liver, adrenal or muscle sections of the untreated, scorbutic guinea pigs, small amounts of glycogen were present in each of these tissues in the animals that received either ACTH or cortisone,¶ even at the time of death from scurvy.

Studies of the adrenal glands. The data on adrenal size are shown graphically in Fig. 2. The four normal animals weighing 270 to 340 g had adrenal-pair weights averaging 145 mg ± 50 mg. The average ratio of adrenal weight in milligrams to body weight in grams—an index of relative adrenal size—was 0.46 ± 0.12. The ascorbic acid content varied from 28.6 to 70.8 μg/100 mg of gland, averaging 51.3 μg. The adrenal glands of the untreated scorbutic animals were considerably hypertrophied, as has been previously described(7), but were otherwise grossly normal in appearance. The adrenal-pair weights were rather uniform, being 300 to 325 mg in 6 of 7 instances, and averaged 315 mg, about twice that of the normals. The gland/body ratio averaged 1.2 ± 0.28, which approximates the relative 250% increase for scurvy described by LaMer(7). The ascorbic acid content of these glands in every case was too low to be measurable.

¶ Acknowledgement is gratefully made for the cooperation and assistance of Drs. J. W. Blunt and R. Lattes in these studies.

In the cortisone-treated group, the adrenal gland size approached that of the normals except in 2 instances in which the weight was 300 and 340 mg, respectively. The average gland/body ratio was 0.74 ± 0.25, intermediate between the normal and the scorbutic controls. The adrenal ascorbic acid values were determined for six animals and found to be less than 2.5 μg/100 mg of fresh gland in four, *i.e.*, too low to be measurable. The other two had values of 3.9 and 4.3 μg/100 mg. In the ACTH group remarkable hypertrophy occurred, with adrenal pair weights varying from 430 to 660 mg, and averaging 550 mg. The gland/body ratio averaged 2.06 ± 0.54, the highest ratio of the experiment. The adrenal ascorbic acid content was under 2.5 μg/100 mg, again too low for measurement.

Morphology. Adrenal hypertrophy has been known to occur in scurvy since LaMer's description in 1920. In the untreated scorbutic animals (Group I), microscopic examination of the adrenals disclosed increased cell size, enlarged vesicular nuclei, and an increased number of cytoplasmic granules. The adrenals of the ACTH-treated guinea pigs showed the same changes but to a more marked degree, with actual mitotic figures occurring not infrequently, and greatly increased granule formation in the cells. The glands of the cortisone-treated group, by contrast, revealed small cells with few, if any, granules, and shrunken dark nuclei; a picture of involution.‖

Discussion. It would appear that the administration of ACTH or of cortisone moderates the course of scurvy in the guinea pig. Animals receiving the hormones were slower to lose weight and to show overt signs of the disease than were controls. Moreover, they lived appreciably longer. Post-mortem examination disclosed, both macroscopically and microscopically, that the treated animals presented relatively less extreme manifestations of scurvy. The finding of Schaffenburg, *et al.*(12), that cortisone exerts a protective action in guinea pigs rendered scorbutic is thus confirmed. However, the observation that a similar action is produced by ACTH makes it necessary to reconsider certain of the inferences that have hitherto been drawn in respect to the relation of ascorbic acid to adrenal function.

Because of the fall in adrenal ascorbic acid after the administration of ACTH(18-21), it has been assumed by many that ascorbic acid is necessary for, and is actually utilized in the production of the corticosteroids. In an unconfirmed report(22) an actual corticosteroid-ascorbate adrenal product has been described. As mentioned previously, Schaffenburg, *et al.*(12), reported on the possibility that "some of the mesenchymal alterations in scurvy were not due to deficiency of vitamin C as such, but resulted from hyposecretion of cortisone-like steroids." They further postulated that compensatory ACTH overproduction occurred in consequence of insufficiency of carbohydrate-active corticosteroids secondary to vitamin C deficiency.

With this viewpoint, the mitigating effect of cortisone on the lesions of scurvy might be explained in terms of sparing ascorbic acid. One might suppose that the decreased activity of the adrenal cortex which follows upon cortisone administration is accompanied by decreased utilization of ascorbic acid. This, in turn, would release a certain amount of the vitamin which could serve to prolong the life of the animal on a scorbutic regimen.

‖ Acknowledgement is made to Dr. J. W. Jailer for assistance in the interpretation.

18. Sayers, G., and Sayers, M. A., *Fed. Proc.*, 1946, v5, 200.

19. Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinol.*, 1946, v38, 1.

20. Long, C. N. H., *Fed. Proc.*, 1947, v6, 461.

21. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinol.*, 1948, v42, 379.

22. Lowenstein, B. E., and Zwemer, R. L., *Endocrinol.*, (abstract), 1946, v39, 63.

However, an inference of the same hypothesis would be that ACTH should exert an opposite effect. For, by stimulating the adrenal, ACTH should accelerate the utilization of ascorbic acid and perhaps hasten the death of the animal rendered scorbutic. In fact, however, the influence of ACTH in scurvy appears to parallel that of cortisone. The intense hypertrophy and increase in the secreting granules of the adrenals indicate almost beyond doubt that corticosteroids were produced in large quantities, and this in spite of virtual absence of adrenal ascorbic acid on assay. The production of corticosteroids in the absence of vit. C had been previously suggested on other grounds, both for the guinea pig(20) and the chick(23). From the clinical side, additional evidence is derived from the observation that rheumatoid arthritis in one patient responded favorably to the administration of ACTH despite the coexistence of clinical scurvy. It is difficult to reconcile these findings with the concept that ascorbic acid constitutes a limiting factor in the synthesis of corticosteroids by the adrenal cortex.

The relation between vit. C and the activities of the adrenal cortex thus remains undefined. It may well be that the corticosteroids somehow influence the metabolism of ascorbic acid in many tissues and perhaps reduce the requirement of the organism for this vitamin.

As for the relation of the experimental findings to the clinically observed remissions of various types of purpura under the influence of ACTH, there is little that can be said without further study. It does appear that by the administration of cortisone or ACTH the hemorrhagic manifestations of scurvy may be made appreciably less severe in the guinea pig as well as in man.

Summary. 1. Cortisone and ACTH prolong life and reduce the hemorrhagic manifestations of scurvy in guinea pigs. The effects of the two hormones are similar. 2. The effect of these two hormones is also parallel in the direction of maintenance of glycogen storage in liver, adrenal, and muscle in the scorbutic guinea pig. 3. The moderating effect of ACTH on scurvy makes it seem probable that vitamin C is not needed for the production of the corticosteroids similar to cortisone. This idea receives further support from other observations, including the demonstration of increased adrenal activity induced by ACTH in the absence of demonstrable adrenal ascorbic acid. 4. The locus of action of adrenal corticosteroids in the amelioration of certain hemorrhagic phenomena is not clear, but may be on the blood vessels or perivascular tissues.

Addendum. Further experience has shown that it is possible to produce severe toxic effects in the guinea pigs with these relatively large doses of cortisone and ACTH so that the protective effect of these hormones then may not occur. Hyperadrenalism, however, is manifested in all the treated scorbutic animals.

The authors wish to thank Misses Barbara Mulliken, Marion Ganzel and Patricia Higbee for technical assistance.

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23. Jailer, J. W., and Boas, N. F., *Endocrinol.*, 1950, v46, 314.

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Effect of Aureomycin on Heparin Concentration and Clotting Time of Human Blood.* (18236)

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While Macht and Farkas(1) reported that aureomycin increased the coagulability of blood, other investigators found no evidence for this following the administration of the drug(2,3). The mechanism of an effect of aureomycin on coagulability may be a reaction between the aureomycin and the naturally occurring heparin. Experiments demonstrating that aureomycin does lower the concentration of heparin in human plasma and serum and also increases the coagulability of blood are presented in this report. Increasing amounts of aureomycin were added to normal serum and plasma in the following manner: Aureomycin† (5 μg to 8 mg) was diluted with about 1 cc of 0.9% NaCl, adjusted to pH 7.3 with 0.1 N-HCl, brought to a volume of 2 cc with 0.9% NaCl and added to 10 cc of clear normal serum or plasma. The mixture was centrifuged for 15 minutes at 1600 times gravity and –2°C. The supernatant was analyzed for heparin‡ by the method of Jacques, Monkhouse, and Stewart(4) with slight modifications to be described in a subsequent report(5).

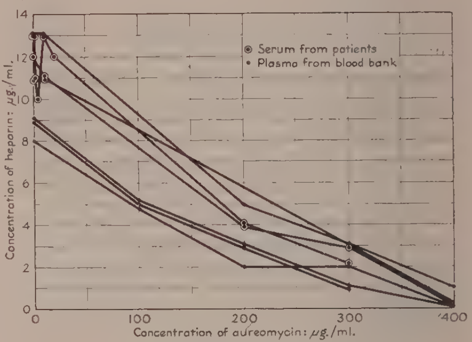

FIG. 1.

Effect of aureomycin on the concentration of heparin in human serum and plasma.

The effect of aureomycin on blood coagulability was investigated by determinations of the coagulation times of 11 patients just before and just after infusions of aureomycin. The coagulation times were done by the same technician using the 3 tube method of Lee and White. After a control coagulation time, 0.5 g of aureomycin dissolved in 500 cc of 0.9% NaCl was administered intravenously. When the infusion was finished the coagulation time of blood drawn from a vein in the opposite arm was determined.

As increasing amounts of aureomycin were added to the serum and plasma *in vitro,* the amount of heparin decreased (Fig. 1). In the presence of 100 to 200 μg aureomycin/cc serum the concentration of heparin was approximately halved. Less than 20 μg aureomycin/cc did not appear to have a significant effect on the concentration of heparin as measured by the method used.

The blood of 10 of the 11 patients clotted more rapidly after an infusion of 0.5 g of aureomycin dissolved in 0.9% NaCl (Table I). In one patient there was no change in the coagulation time. There was no instance in which the coagulation time was increased after the infusion of aureomycin.

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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2. Harned, B. K., Cunningham, R. W., Clark, M. C., Cosgrove, R., Hine, C. H., McCauly, W. J., Stokey, E., Vessey, R. E., Yuda, N., SubbaRow, Y., *Ann. N. Y. Acad. Sci.,* 1949, v51, 182.

3. Ross, S., Burke, F. G., Rice, C. C., Schoenback, E. S., Bischoff, H., Washington, J. A., *Clin. Proc. Child. Hosp.,* 1948, v4, 315.

† Kindly supplied by Lederle Laboratories.

‡ Heparin of Hynson, Westcott and Dunning, Inc., used for standards.

4. Jacques, L. B., Monkhouse, F. C., Stewart, M., *J. Physiol.,* 1949, v109, 41.

5. Ochs, M., M. S. Thesis in Physiological Chemistry, University of Minnesota, in preparation.

TABLE I. Effect of Aureomycin Given by the Intravenous Route on Coagulation Time.

Patient No.	Clotting time (min.) Before aureomycin	After aureomycin	Diff.
1*	30	19.5	—10.5
2	21	15	— 6
3	19	17	— 2
4	19	17.5	— 1.5
5	17	13	— 4
6	22	15.5	— 6.5
7	23	19.5	— 3.5
8	23	21	— 2
9	26	23	— 3
10	23	23	0
11	16	12	— 4

* Convalescing from infectious hepatitis.

The data presented is in accord with Macht and Farkas' finding(1) that in certain instances aureomycin increases the coagulability of the blood. The present results suggest that this increased coagulability is due to an anti-heparin effect of aureomycin. The observation of Miller *et al.* that certain antibiotics including aureomycin reduce mortality from x-radiation(6) and the finding of Allen *et al.* that shortly after x-radiation a heparin-like substance appears in the plasma of dogs (7), suggest the possibility that protection by certain antibiotics against the effects of radiation may be mediated, at least in part, through an anti-heparin effect. Whether or not this phenomenon is responsible for an increased incidence of thrombosis following the use of antibiotics, which has been suggested by some observers and denied by others(8), should be investigated further. It is worthy of note that both streptomycin and penicillin have been shown to increase the coagulability of blood(9-11).

Summary. Addition of aureomycin to human plasma *in vitro* produces a significant decrease in the heparin concentration. Aureomycin administered by the intravenous route lowered the coagulation time of the blood of 10 of 11 patients.

The authors are grateful for the technical assistance of Frances Simons and Mary J. Ochs.

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Effects of X-irradiation on Passively Transferred Antibody.* (18237)

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Although much work has been done in an attempt to elucidate the mechanisms involved in antibody production and the factors which influence active immunity, very little is known about the mechanism of antibody destruction. Heidelberger *et al.*(1) have demonstrated that dietary amino acids containing isotopic nitrogen (N 15) are incorporated into actively produced antibody in similar quantities to their incorporation into the plasma proteins, and that the rate of disappearance from the 2 proteins is similar. Passively transferred homologous antiserum showed no uptake of the dietary isotopic nitrogen and was therefore considered anabolically inert.

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* This project has been supported by the Anna H. Hanes Fund.

† Address after July 1, 1950: Dept. of Hematology, Barnes Hospital, St. Louis, Mo.

1. Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, 1942, v144, 555.

TABLE I. Rate of Disappearance of Passively Transferred Homologous Antibody.

	Time in days after injection of antiserum								
	1/24	1	2	3	4	6	8	10	12
Irradiated	1333	1000	640	600	600	400	160	75	60
	800	640	600	400	300	160	100	60	60
	1000	800	600	400	300	160	120	60	60
Control	1333	533	400	400	400	200	120	75	40
	1600	800	400	400	400	300	100	60	60
	1600	1000	400	600	400	200	100	60	40

TABLE II. Rate of Disappearance of Passively Transferred Heterologous Antibody.

	Time in days after inj. of antiserum					
	1/24	1	2	3	4	6
Irradiated	2132	800	400	200	160	40
	1600	800	400	160	160	80
	1600	533	266	200	160	20
Control	1600	800	400	160	40	20
	1600	800	266	160	80	20
	1600	800	200	160	80	40

X-irradiation has been shown to inhibit antibody formation(2). Once active antibody formation has started, however, irradiation does not alter the response of the animal to the antigen(2,4). It seemed desirable, therefore, to study the effect of x-ray on the rate of antibody destruction after passive transfer. In our experiments both heterologous and homologous antisera were used, since Glenny and Hopkins(3) have shown that the former is destroyed much more rapidly than the latter.

Materials and methods. Albino rabbits from 2-2.5 kg in weight were used in all experiments. The antisera were given intravenously 24 hours after irradiation, and control animals were injected at the same time. Blood samples for antibody titer were taken thereafter at intervals as shown in Tables I and II. The homologous antiserum was a rabbit anti-sheep red blood cell hemolysin with a titer of 1:5333, and 25 cc of the antiserum was injected intravenously into each animal. Hemolysin titers were determined by a standard technic, with excess complement and 2% sheep RBC's as indicator. The end point was taken as the last tube showing clear sparkling hemolysis after 1 hour in a water bath at 37°C. The heterologous antiserum was obtained from a patient recovering from brucellosis, and 7.5 cc of this antiserum with a titer of 12,500 was injected intravenously. Agglutinin titers were determined with equal parts of diluted serum and a heat killed antigen after 24 hours in a water bath at 56°C and 24 hours refrigeration at 8°C. Individual tube dilutions, rather than serial dilutions, were used in all experiments.

X-ray dosage was patterned after the method of Craddock and Lawrence(4), who reported that a dosage of 250 r was sufficient to depress antibody production in rabbits without producing changes in the peripheral blood or general nutrition of the animals. We used a total of 300 r with the following radiation factors: 250 Kv, 18 ma, 0.5 mm Cu and 1 mm Al filters, 50 cm distance, open port, and 72 r/min. dose rate. This small roentgen dosage was felt to be preferable in order to prevent, as much as possible, the protein catabolic effects of irradiation sickness. With the animal stretched on a board, the effective radiation, as checked with a fluorescent screen, did not include small portions of the extremities and of the ears.

Twenty-five days after the initial serum injection, anti-human serum titers were determined on the animals which had received the human brucella immune serum, using the same human brucella serum as the antigen. The undiluted rabbit serum was placed in precipitin tubes and overlayed with the antigen in dilutions of 1:5, 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000. The last tube

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2. Hektoen, L., *J. Infect. Dis.*, 1919, v17, 415.
3. Glenny, A. T., and Hopkins, B. E., *J. Hyg.*, 1922-23, v21, 142.
4. Craddock, C. G., Jr., and Lawrence, J. S., *J. Immunol.*, 1945, v60, 241.

showing reaction at the interphase after 20 minutes was taken as the end point.

Results. As can be seen in the accompanying tables, the rate of destruction of the homologous antibody (Table I) and the heterologous antibody (Table II) was markedly different and fell within the ranges described by Glenny and Hopkins(3). There was no difference between the irradiated and the control animals. The anti-human serum titers were determined primarily to check the efficacy of this irradiation method and dosage in inhibiting antibody formation. Results in the 3 irradiated rabbits were positive in antigen dilutions of 1:5, 1:10, and 1:10 respectively; the 3 control animals were all positive in 1:1000 antigen dilution.

Summary and conclusions. X-irradiation in dosage of 300 r had no effect on the rate of destruction of passively transferred homologous or heterologous antisera given intravenously to rabbits 24 hours after irradiation. This x-ray dosage was sufficient, however, to decrease, but not totally prevent, the formation of antibody to the heterologous serum proteins.

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Influence of Fasting on the Effect of Insulin on Glucose Oxidation.* (18238)

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It has generally been believed that insulin increases the immediate oxidation of glucose and this is frequently stated in text books(1). However as Soskin and Levine(2) point out in their monograph, "this is without basis in fact." There has been no good experimental evidence that insulin will increase the oxidation of glucose until recently(3) when by using carbon^{14}-labeled glucose it was found that the old assumption is correct. Insulin actually does increase the oxidation of glucose by the extra-hepatic tissues to carbon dioxide and water. This report concerns the influence of fasting on the effect of insulin on glucose oxidation by the extra-hepatic tissues.

Experimental. The general plan of the experiments and the details of the methods used have been given elsewhere(3-6). After varying periods of fasting male rabbits (average weight 2 kg after evisceration) were eviscerated(7) and given an injection of a priming dose of carbon^{14}-labeled glucose so as to make the specific activity of the body glucose the same as the glucose to be used for the constant injection. With the aid of frequent blood sugar determinations the blood sugar was maintained at a normal level (70-130 and as near 100 mg % as possible) for 8 or more hours by the constant injection of carbon^{14}-labeled glucose. Ten units of insulin† were given intravenously each hour. Collection of the expired carbon dioxide was commenced immediately after the injection of the radioactive glucose.

Results. The data in Fig. 1 were calculated

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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† We are indebted to Eli Lilly and Co. for generous supplies of (Iletin) insulin.

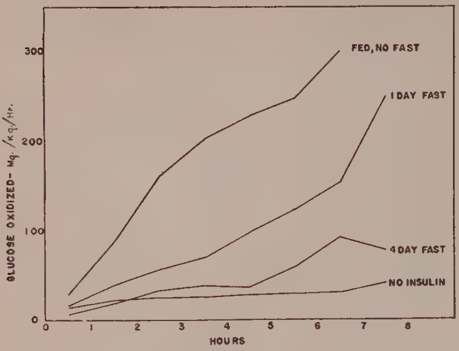

FIG. 1.

Influence of fasting on effect of insulin on glucose oxidation in the eviscerated rabbit. Three of the rabbits were given insulin. The rabbit which received no insulin had been fasted for one day.

from the specific activity (radioactive counts per minute per mg carbon) of the expired CO_2 and injected glucose. The rate of glucose oxidation increases rapidly with time after the completion of the evisceration and the simultaneous beginning of insulin administration. It is obvious that a single day of fasting greatly reduces the extent of insulin action on the rate and amount of glucose oxidized. A 4-day fast reduces the insulin action still more so that except for the last 3 of the 8 hours of observation the oxidation of glucose is little greater than when no insulin is given. When insulin is not given fasting has relatively little influence on the rate of glucose oxidation. The average glucose oxidation for the 8 hours of observation was with insulin and no fast, 199; 1-day fast, 114; 4-day fast, 52 and without insulin 30 mg per kg per hour. The usual effect of fasting(7,8) on glucose disappearance ("utilization") is noted. Glucose "utilization" with insulin and no fast was 660; 1-day fast, 540; 4-day fast, 258 and without insulin 112 mg per kg per hour.

Discussion. The literature on the influence of fasting and/or the diet upon sugar tolerance and the action of insulin is well reviewed by Lundbaek(9). Drury(7) and Bergman and Drury(8) have demonstrated the influence of fasting on the glucose "utilization" (glucose required to keep the blood sugar normal) of the eviscerated rabbit. Since these animals had no pancreas this would seem to dispose of the theory of Best, Haist and Ridout(10) who believe that the insulin content and output of the pancreas was decreased by fasting or dietary carbohydrate deprivation. Himsworth(11-13) first suggested that the decreased glucose tolerance and insulin sensitivity resulting from fasting or a low carbohydrate diet was due to the reduced production of a normal activator of insulin. Later experiments led him to conclude(14) that the effect of fasting was due to the increased production of an inhibitor of insulin which presumably arises in the anterior pituitary. There is other evidence however(15-18) which suggests that the hypophysis is unnecessary for the effect of diet and fasting on sugar tolerance and insulin sensitivity.

Insulin action may be studied with much greater certainty through its effect on the *oxidation* of glucose. The influence of the hormones of the anterior pituitary and adrenal cortex in this connection would be of much interest in relation to fasting.

Summary. Insulin increases the rate of glucose oxidation in the eviscerated rabbit. The influence of insulin upon glucose oxidation is most marked in the non-fasted fed animal. It is less effective after a day's fast prior to evisceration and the effect is greatly decreased by a still longer fasting period.

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Effect of Beryllium on *In Vitro* Calcification of Cartilage. (18239)

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"Beryllium rickets"(1-6) has generally been ascribed to the marked hypophosphatemia resulting from diminished absorption of phosphate from the gastro-intestinal tract due to precipitation of beryllium phosphate in the gut. There are indications, however, that beryllium also adversely affects the so-called local factor in calcifying cartilage(4). Thus administration of vitamin D will restore the serum inorganic phosphate to normal levels but nevertheless appears to be incapable, even in high dosage, of wholly preventing or curing the abnormality in endochondral calcification(4,5). Moreover, Sobel(4,7) found that the cartilage of rats fed a beryllium-containing diet would calcify only very imperfectly *in vitro* in solutions containing adequate concentrations of calcium and inorganic phosphate. This latter finding is of interest in connection with recent evidence(8-12) that beryllium in very low concentration markedly inhibits alkaline phosphatases, being more selective in this respect than cyanide(13-15), formaldehyde(16) or any other chemical or physical agent yet employed. The aim of the present study was to exploit this property of beryllium for further investigation of the role of enzymes, in particular alkaline phosphatase, in endochondral calcification.

Methods. Twenty-day-old rats were placed on the Schneider-Steenbock rachitogenic diet R-14 for 7-10 days to produce moderately advanced rickets and then sacrificed. For our preliminary studies of Be inhibition of cartilage alkaline phosphatase activity, the epiphyseal cartilage of the tibiae and femora of 2 rats was dissected out for each experiment, ground with sand in 10 ml 0.04 M veronal buffer, pH 9.8, and the homogenate then centrifuged. The phosphatase activity of the samples was then standardized by diluting the slightly turbid supernatant with veronal buffer (usually 3-5 ml supernatant diluted to 10 ml) to give enzyme activity sufficient to split off 10-20 γ P as inorganic phosphate by a 0.2 ml sample after one-half hour incubation with 1% sodium β-glycerophosphate. In experiments to determine percent inhibition by various molarities of beryllium, 0.8 ml beryllium sulfate in concentrations prepared to give the indicated final molarities was added to tubes containing 0.2 ml standardized cartilage extract and 1.0 ml β-glycerophosphate-veronal buffer-substrate solution. The mixture was incubated for 30 minutes at 37°C. (Control tubes were similarly prepared but without added beryllium). After cooling in an ice-bath, 2 ml 10% trichloroacetic acid was added and inorganic P determined by the Fiske and Subbarow method adapted to photoelectric colorimetry. Time-activity experiments were carried out in the same way except for varying the time

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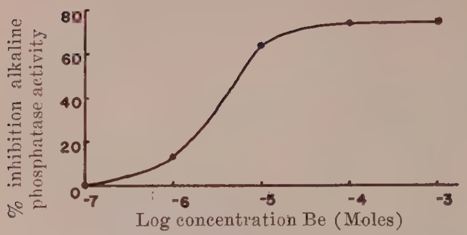

FIG. 1.

Percent inhibition of cartilage alkaline phosphatase activity by Be in various concentrations. (Buffer - substrate: β-glycerophosphate - veronal; final pH 9.6; time ½ hr; temp. 37°C).

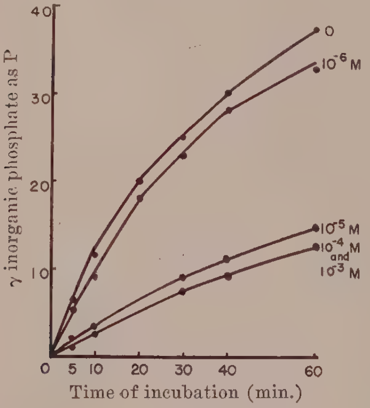

FIG. 2.

Time-activity curves for inhibition of cartilage alkaline phosphatase activity by Be in various concentrations. (Buffer-substrate: β-glycerophosphate-veronal, final pH 9.6; temp. 37°C).

of incubation from 5-60 minutes. The data incorporated in Fig. 1 and 2 represent the means of 8 and 3 sets of runs respectively. For the *in vitro* calcification experiments, contiguous slices of the proximal ends of moderately rachitic rat tibiae were used in controlled sets of experiments conducted as previously described(17). The basal salt solution employed contained potassium chloride 50 meq., sodium chloride 700 meq., sodium bicarbonate 220 meq. per liter, no added magnesium. The calcifying solutions contained in addition Ca (10 mg % as calcium chloride) and P as inorganic phosphate (6 mg %) or as phosphoric ester of the type and in the amount specified in the text. Beryllium sulfate was added to the appropriate calcifying tubes in amounts sufficient to give the indicated final molarities. The pH of the incubating solutions was adjusted to 7.4, checking with a Cambridge glass electrode pH meter. The slices were incubated 18-24 hours at 37°C, then stained with 3% silver nitrate. The results reproduced in Fig. 3 are representative of 5 uniform experiments of each type illustrated.

Results. Inhibition of cartilage alkaline phosphatase activity by beryllium. As indicated in Fig. 1, beryllium in concentrations of 1.0 x 10^{-5}M or greater markedly inhibits cartilage alkaline phosphatase activity, approximately 70% inhibition being noted at the end of the half-hour incubation period. In concentrations of 1.0 x 10^{-6}M beryllium, inhibition is sharply decreased, to a mean of approximately 12% under the conditions of the experiment, and at lower Be concentrations inhibition is negligible. These results with rat cartilage alkaline phosphatase are in good agreement with those reported by Klemperer *et al.*(9) for the range of beryllium concentrations effective in inhibiting hog kidney alkaline phosphatase. The data are also in general agreement with those reported by Grier *et al.*(10) for the effects of various molarities of Be on rat bone, sarcoma and intestinal alkaline phosphatases, although their figures suggest a more uniform slope in Be concentration - phosphatase inhibition curves than indicated by the data of Klemperer *et al.*(9) and of the present authors.

Fig. 2 is a time-activity curve which again emphasizes the critical difference in inhibition of cartilage alkaline phosphatase activity between the ranges 1.0 x 10^{-6}M and 1.0 x 10^{-5}M Be. It is apparent from these curves that enzyme inactivation occurs within the first 5 minutes of incubation, probably because of replacement of Mg by Be, according to evidence suggesting competition between these 2 ions(9,10).

Effect of beryllium on in vitro *calcification with phosphoric esters. In vitro* calcification experiments were carried out with β-glycerophosphate as the sole source of phosphorus,

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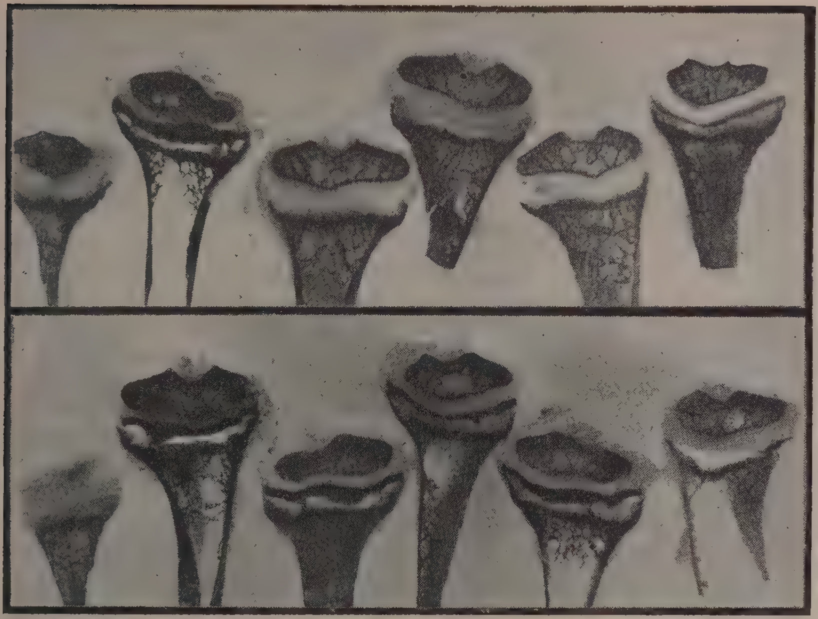

FIG. 3.

Top row, from left to right: 1. Control slice incubated in basal salt solution (B.S.S.) without Ca or P; note wide zone of uncalcified cartilage. 2. Control slice incubated in B.S.S. + Ca + β-glycerophosphate (10 mg % P); note good calcification. 3,4,5,6. Slices incubated in B.S.S. + Ca + β-glycerophosphate (10 mg % P) + Be 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, respectively. Note complete inhibition of calcification in slices 3, 4, and 5, but definite calcification with only partial inhibition in slice 6.

Bottom row, from left to right: 1. Control slice incubated in B.S.S. without Ca or P; note wide zone of uncalcified cartilage. 2. Control slice incubated in B.S.S. + Ca + inorganic P (6 mg %); note good calcification. 3,4,5,6. Slices incubated in B.S.S. + Ca + inorganic P (6 mg %) + Be 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, respectively. Note no inhibition of calcification in slices 3, 4, and 5, but complete inhibition in slice 6.

in concentrations equivalent to 10 mg % P, in the absence of beryllium and in the presence of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M Be. Good calcification obtained with β-glycerophosphate was completely inhibited in all dilutions of added beryllium up to 10^{-6}M (Fig. 3). In the presence of 1.0×10^{-6}M Be, definite calcification was noted although some inhibition was present (Fig. 3). Similar results were obtained when sodium phenylphosphate, in concentrations equivalent to 10 mg % P, and creatine phosphate, in concentrations equivalent to 20 mg % P, were present as the sole source of phosphorus. Calcification was inhibited completely, or virtually so, in all dilutions of beryllium up to 10^{-6}M, in which concentration inhibition was slight and definite calcification was noted. *In vitro* calcification experiments with β-glycerophosphate in higher concentration, equivalent to 20 mg % P, revealed that increasing the substrate tended to decrease the inhibitory effect of beryllium; there was no indication of inhibition by 10^{-6} or 10^{-5}M Be but there was definite inhibition by 10^{-4} and complete blocking by 10^{-3}M Be. That increasing concentrations of phosphoric ester substrate decrease beryllium inhibition of dephosphorylation by alkaline phosphatase was noted by Klemperer(9) using various concentrations of phenylphosphate.

Effect of beryllium on glycolysis of cartilage. Manometric studies of glycolysis by rat cartilage homogenates in Ringer-bicarbonate solution indicated no inhibition by beryllium in concentrations of 1.0×10^{-3}M. This finding is consistent with the report by Klemperer (2) that glycolysis by acetone liver or kidney preparations is not inhibited by beryllium in concentrations up to 10^{-3}.

Discussion. There is a striking correspondence between the concentrations of beryllium which markedly inhibit cartilage alkaline phosphatase and those required to block *in vitro* calcification of cartilage when phosphorus is supplied as β-glycerophosphate, phenylphosphate or creatine phosphate. This finding is consistent with Robison's theory of the role of alkaline phosphatase in making the phosphorus of phosphoric esters available for calcification of cartilage(18). On the other hand, it is difficult to reconcile with the Robison theory the failure of beryllium (except in concentrations of 10^{-3}M) to inhibit *in vitro* calcification of cartilage when phosphorus is supplied as inorganic phosphate. There are other indications that the enzyme mechanisms involved in *in vitro* calcification of cartilage are not the same when the source of phosphorus is β-glycerophosphate, phenylphosphate or creatine phosphate as when phosphorus is supplied as inorganic phosphate. Thus, 10^{-3}M iodoacetate, 10^{-4}M fluoride and 10^{-2} phlorizin, which do not inactivate alkaline phosphatase, do not inhibit *in vitro* calcification of cartilage with β-glycerophosphate, phenylphosphate or creatine phosphate(17, 19,20) but markedly inhibit calcification with inorganic phosphate(17-19). These effects of phlorizin, iodoacetate and fluoride, well known inhibitors of glycogenolysis, form the basis for the view(17,19,20) that phosphorylative glycogenolysis is an integral part of the complex processes of calcification of cartilage in a medium containing phosphorus as inorganic phosphate in physiological concentrations. According to this concept the role of alkaline phosphatase in the hypertrophic cartilage cell does not at present appear to be essential for calcification, at least as a dephosphorylating enzyme, and remains obscure(20).

It will be noted that *in vitro* calcification of cartilage with inorganic phosphate was completely inhibited by beryllium in concentrations of 10^{-3} (Fig. 3). In this concentration beryllium can no longer be regarded as a highly selective inhibitor of alkaline phosphatase. We cannot, however, ascribe the effect on calcification with inorganic phosphate to inhibition of the glycolytic system of enzymes in cartilage and the enzyme system blocked by 10^{-3}M Be remains to be identified.

In regard to the nature of "beryllium rickets," our *in vitro* calcification experiments bear out the contention of Sobel, Goldfarb and Kramer(4) that beryllium interferes with the function of the local factor. The nature of this interference remains obscure but presumably some enzyme essential for calcification is inhibited. Our studies suggest that inhibition of cartilage alkaline phosphatase will not satisfactorily account for the failure of calcification under these circumstances.

Summary. 1. Beryllium in concentrations of 1.0×10^{-5}M or greater is a potent inhibitor of the dephosphorylating activity of cartilage alkaline phosphatase. 2. In concentrations of 10^{-5}M or greater, beryllium completely blocked *in vitro* calcification of cartilage when phosphorus was supplied as β-glycerophosphate, phenylphosphate or creatine phosphate. 3. When phosphorus was supplied as inorganic phosphate (the condition apparently obtaining *in vivo*), concentrations of beryllium sufficient to produce marked inhibition of alkaline phosphatase failed to interfere with *in vitro* calcification of cartilage. 4. The significance of these findings in relation to current theories of endochondral calcification and "beryllium rickets" is discussed.

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Acute Necrosis and Malformation of Developing Mammalian Brain Caused by X-Ray.* (18240)

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The nervous system of the adult in contrast to that of the embryo is considered to be fairly resistant to ionizing radiation except in so far as it may be damaged secondarily by changes in its blood vessels(1). Lymphoid and hemopoietic tissues and cells in mitosis are usually classed as the most susceptible elements but there is little specific information concerning the vulnerability of developing mammalian nervous tissue. That neuroblasts might be radiosensitive has been indirectly suggested by the therapeutic effects on neurogenic embryomas such as neuroblastoma and medulloblastoma; also, it is widely believed that microcephaly and other failures of development of the human nervous system may follow irradiation of the embryo *in utero*(1). A more concrete form of evidence is given by the experiments of Rugh(2) who showed that the neuroblasts in developing lower animal forms such as Amblystoma, are the most sensitive cells to irradiation. The retinas in newborn mice and humans have been shown to be damaged by x-radiation(3), and gross malformations of skeletons of rats irradiated *in utero* have been observed by Russell(4).

In the course of an investigation of the pathologic effects of ionizing radiation upon the development of mammalian nervous system it was found that growing central nervous tissues in fetal rats and mice could be selectively and extensively destroyed by x-radiation with virtually no destructive effect on other organs. When the pregnant animal was given total body irradiation of 200-600 r her fetuses developed severe destruction of the developing nervous tissue in a few hours. After 150 r damage was less and fetuses allowed to come to term and grow up showed severe malformations of the brain.

Methods. Essentially the experiments consisted in administering total body radiation to pregnant white rats, C3H and C57 mice at varying stages of gestation. Usually this was done in the second and third week so that the fetuses were already fairly well developed. Irradiation was carried out at 35, 50, 75, 100, 150, 200, 400 and 600 r in single exposures. The fetuses were removed and sacrificed for pathologic study at intervals of 30 minutes, 2, 4, 24, 48 and 96 hours after irradiation, or they were allowed to go to term when they were studied at ages of one day to several months. To date, about 20 litters have been obtained for study from animals irradiated at 100 r or below, 10 litters at 150 r, 4 litters at 200 r, 15 litters at 400 r, and one litter at 600 r. In addition several litters of rats were irradiated (total body) one to 4 days after birth at 100, 150 and 200 r and studied at varying intervals after the radiation. Fixation of the freshly sectioned tissues was done in Bouin's fluid, embedded in paraffin, and examination chiefly with hematoxylin and eosin stains. Bodian protargol and phosphotungstic hematoxylin

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* This investigation was carried out under contract AT(30-1)-699 with the Atomic Energy Commission through the Harvard Medical School, Boston.

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stains were sometimes used. Fetuses were examined whole by interrupted serial frontal or lateral sections; older animals were completely autopsied and all organs examined histologically. The brain was studied in interrupted serial frontal sections.

Irradiation was delivered from General Electric Maximar machines at 200 Kv, 10 milliamperes and without added filter. The machines had an inherent filter of 3 mm of aluminum. The rats were irradiated in thin-walled flat cardboard containers, on a wood table. Mice were irradiated in a fiberboard flat cage with a copper screen top. The target distance was measured to the table, that is to the lowermost part of the animals which were obliged to lie flat by the narrow height of the respective containers. The target distance was 64 cm for rats and 44 cm for mice. The amount of radiation under these circumstances was estimated in air and checked at frequent intervals with a Victoreen condensor type ionization chamber placed in the empty containers. This measurement included the back-scatter from the containers and table. As checked with the Victoreen, variations of only a few r occurred from one experiment to another. The true error, as in any modern radiation technics, cannot be exactly measured, so that the figures 150 r, 400 r, etc., are approximations based on the Victoreen readings.

Results. Irradiation at 200, 400 and 600 r produced severe and often widespread destruction of the rapidly growing regions of the brain in all fetuses and usually of the retina, cord and some ganglia. This was complete in 24 hours, well developed in 6 hours, and clearly beginning in two hours after irradiation. *No difference in sensitivity between mouse and rat fetuses was apparent.* At 600 r the thymus showed severe necrosis, and there were rare foci of necrosis in other tissues as noted below. At 400 r there was rarely scant damage outside of the nervous system and at 200 r none was seen. At 150 r the brain and usually the cord and retina were damaged but to a lesser degree than at the higher doses. At 35 to 100 r necrosis did not occur except in slight degree in a single animal (100 r). In a given litter the neural lesions were remarkably uniform in pattern and such variations as were seen among different litters appeared to be related to the age of the fetuses; for example fetuses near term usually showed minimal cord lesions.

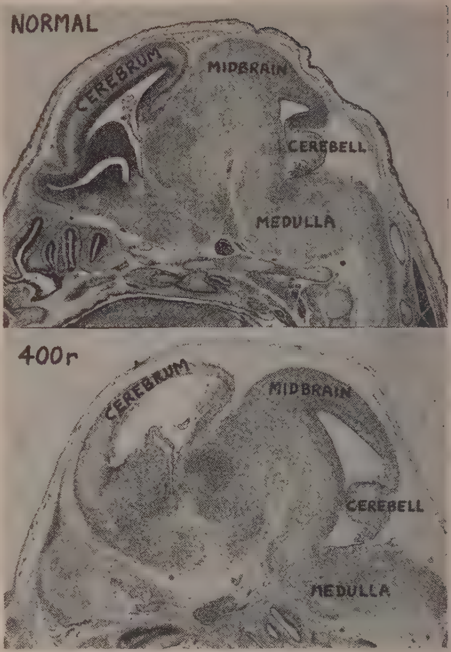

Fig. 1.
Acute radiation necrosis of fetal mouse brain. Above, lateral view of normal in third week of gestation, to show dark-stained zones of densely packed periventricular neuroblasts. Below, necrosis with edema of these zones 24 hr after 400r. (H & E, low power).

Description of the lesions may be conveniently put into arbitrary categories as they were studied: (1) the acute stage of necrosis during the first day following radiation, (2) proliferative and beginning malformative changes two to several days after radiation, (3) late maldevelopments seen in the postnatal period, and (4) extraneural lesions.

Acute stage of necrosis: The acute destructive lesions affected principally the periventricular masses of germinal cells and neuroblasts which are forming the striatal and thalamic masses during the latter two weeks of gestation. Similar regions of rapid growth

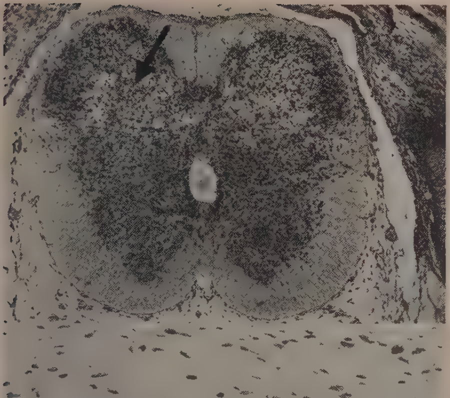

Fig. 2. Top.
Acute radiation necrosis of fetal mouse spinal cord, 24 hr after 400r. (H & E, ×45).

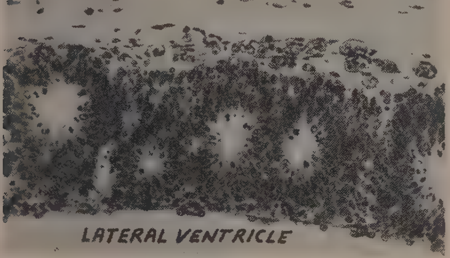

Fig. 3. Bottom.
Early malformation of neuroblast layer following radiation injury. Fetal rat cerebrum 48 hr after 150r, showing "rosette" proliferation of neuroblasts. Mitotic figures are abundant. (H & E, ×133).

were also severely affected in the middle cerebral cortical zones, olfactory lobes, the hippocampus, anterior hypothalamus, and dorsal midbrain just anterior to the cerebellum (Fig. 1). Scattered necrotic cells occurred also in the midbrain and medulla. In younger animals the dorsal gray columns of the spinal cord were damaged often severely (Fig. 2), while near term the cord was largely spared even at 400 r. In older fetuses and those irradiated just after birth where the cerebellum was developing rapidly the outermost cortical layer of neuroblasts was nearly completely destroyed. In a few instances necrosis of developing ganglia at the neuroblastic stage were seen. Mature cells of ganglia, cerebral cortex, cerebellum, etc., were unaffected. Ependymal cells were often spared but sometimes they disappeared with the liquefaction necrosis that was so severe in the subependymal periventricular zones. In the retina the neuroblastic layer was consistently damaged at 200 r and sometimes completely destroyed at 400 r. At 150 r there was usually damage but occasionally it was absent; thus the retina was slightly more radioresistant than the periventricular cerebral regions. In animals that happened to be born hours after irradiation and those irradiated soon after birth at 150 r or more, necrosis occurred in the periventricular subependymal forebrain zones where neuroblasts were still abundant. The outer cerebellar cortical layer was similarly damaged, the cord spared and retina moderately damaged. In the few early fetuses—probably 8 to 10 days old—necrosis of the germinal cells lining the primitive ventricles and "neural tube" was seen.

The earliest evidence of necrosis developed 2 hours after irradiation. There was eosinophilia of the cytoplasm and smudging of the chromatin pattern of those neuroblasts that were in mitosis. In addition there was a scattering of polymorphonuclear leukocytes in the damaged zones and slight capillary congestion. Scant patchy edema had also developed. In 4 to 6 hours necrosis of many of the cells was evident, and by 24 hours whole areas of liquefaction necrosis had developed in which floated cellular detritus and polymorphonuclear leukocytes. By this time there were also a fair number of large phagocytes containing cellular fragments, and sometimes red cells when minute hemorrhages occasionally had developed. The phagocytes seemed, in some instances, to originate from interstitial subependymal and vascular adventitial cells. Mitotic activity in surviving neuroblastic and germinal cells was not stopped. Choroid plexus was undamaged. The striatal white matter adjacent (dorsal and rostral) to the developing zone of neuroblasts was usually partially or completely necrotic. Whether this was death of fibers resulting secondarily from dead young neurons or due to a direct action on the fibers remains to be determined.

Stage of proliferation and early malformation: Animals from a number of litters were

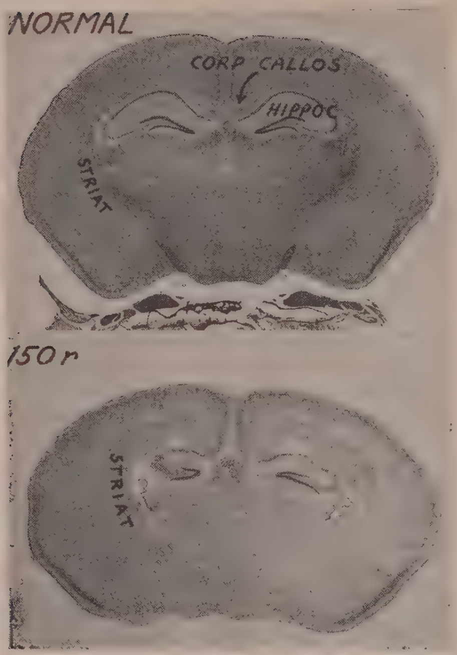

Fig. 4.
Late malformation of brain of mouse, about 3 weeks old, resulting from 150r irradiation about a week before birth. Above, brain of a comparable normal mouse, 3 weeks old. (H & E, low power).

studied 48 hours to several days after irradiation, especially at 150 r. In addition to the patchy necrosis, within 48 hours a bizarre proliferation of the remaining neuroblasts and germinal cells developed characterized by the formation of rosettes and small primitive ependymal canals (Fig. 3). This occurred in brain and cord, especially after 150 r, and was frequently the residual picture in the retina at 200 and 400 r. The pattern resembled that seen in some retinoblastomas and neuroepitheliomas.

Late maldevelopments: Animals from litters exposed *in utero* to 150 r and 200 r in the third week of gestation were found to have severe malformations of the brain when studied from a few days after birth up to the age of 3 weeks (Fig. 4 and 5). Despite their rather grotesque brains they were able to nurse, crawl, then eat, climb, run and bite, but their motor patterns seemed more jerky than normal controls. Detailed neurologic studies were not carried out. Also the animals were sometimes slightly smaller than comparable normal animals. Detailed studies of the skeleton were not made. The malformations seen thus far have been of the following general pattern, and need only be superficially described at present. The corpus callosum is virtually absent with an associated jumbling of the junction of the pyramidal layer of the hippocampus with the medial cerebral cortex. Spotty defects in the outer cerebral cortical neuron layers are seen. The hippocampus is small, its cells patchily jumbled. The striatum—caudate, putamen, globus pallidus and the incorporated bundles of fibers—is incompletely formed; the fiber bundles are nearly absent, the gray masses small and displaced upward. The ventricles are irregular, malformed and dilated. The white matter of the cerebellum is smaller in bulk than normal, and in animals irradiated just after birth the granular layer of the

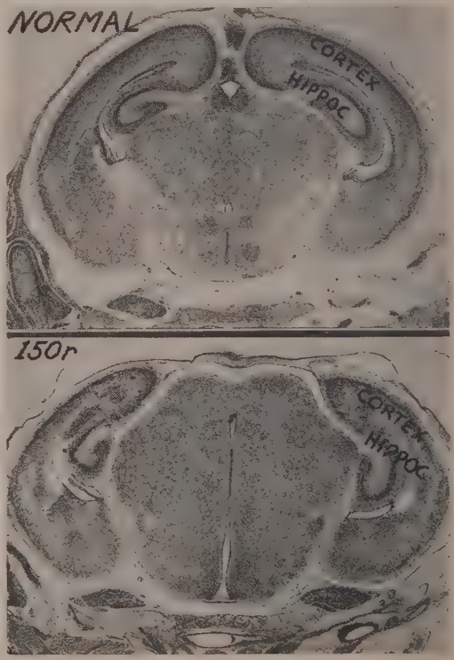

Fig. 5.
Late malformation of brain of mouse about 10 days old, resulting from 150r irradiation about a week before birth. Above, a comparable normal brain (H & E, low power.)

cerebellar folia is absent or reduced in volume. Retinal malformations (after 150 r) are limited to occasional "rosettes" in the rod cell layer. Details of midbrain and cord damage have not been worked out as yet.

Extraneural lesions: As noted, at 600 r a litter of fetuses showed thymus necrosis after 24 hours. In addition rare scattered foci of necrosis were found in the liver, kidney, adrenal and bone marrow. Osteoclasts showed pyknosis of nuclei and some cytoplasmic vacuolization. The blood-forming cells in the liver showed somewhat pyknotic nuclei but destruction was not evident. At 400 r, the thymus was unremarkable, rare foci of necrosis occurred in the marrow in some fetuses, osteoclastic changes appeared rarely as noted above, and in some younger animals necrosis of the outer layer of cells on the tips of the toes and the tip of the genital tubercle were occasionally seen. In one animal rare necrotic muscle fibers in the tongue were seen. After 200 r or less, extraneural lesions were absent.

Discussion. The reasons for the acute radionecrosis in the fetal central nervous system are obscure, but the cells in the areas affected are not only rapidly multiplying but also rapidly growing and differentiating. Protein synthesis is especially active in developing nerve cells(5). It remains to be determined how much of this susceptibility may be influenced by the type of metabolism obtaining at this period of development(6).

In regard to the malformations of the brain evident after birth the important fact is that they occur, and that the developing brain is by far the most radiosensitive tissue in the rat and mouse fetus in the latter half of gestation. At this early stage of the study descriptions of the malformations must be superficial and limited. Additional experiments with radiation given at more definitely known periods of gestation should make the malformations more susceptible to analysis in terms of stages of embryonic development.

The results of these experiments may provide useful tools for the histochemical and biochemical studies of the nature of radiation reaction and neuropathologic developmental anomalies. Investigation of the first and third of these is in progress.

Summary. Irradiation of pregnant rats and mice with 200 to 600 r to the whole body resulted in acute necrosis of the rapidly growing parts of the brain, spinal cord and retinas of their fetuses. This developed within a few hours, was highly selective for the central nervous system, and was often severe and extensive at 200 to 600 r. At 150 r damage was less, and was followed in two days by malformations in the neuroblastic zones. Fetuses allowed to go to term and sacrificed after days or weeks showed severe maldevelopments of corpus callosum, hippocampus, striatum and cerebral cortex. Extraneural lesions were absent below 200 r, rare at 400 r. Below 100 r no destructive lesions occurred.

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Effect of Aminopterin on Animals Receiving Myelokentric or Lymphokentric Acid. (18241)

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In the past 2 years much has been written concerning the effect of Aminopterin on normal and leukemic cells(1). The remissions which occur in acute leukemia, especially acute lymphoid leukemia in childhood, are now well known. Because of this work it seemed to us of interest to discover whether this substance could in any way alter the pathologic changes we have obtained with myelokentric and lymphokentric acids(2,3).

Material and methods. Guinea pigs were given daily intramuscular injections of crude

TABLE I.

Animal No.	Aminopterin, total dose, mg	Teropterin, total dose, mg	Crude myelokentric acid (liters)	Crude lymphokentric acid (liters)	Result	Liver	Spleen	L.N.	Bone marrow	Adrenal
1	4		4		++M	++	++		+++ H	+
2	4		4		++M	++	++		+++ H	+
3	16		4		±M	—	+F		+++ HA	+
4	16		4		±M	—	+F		+++ HA	+
5	22		4		+M	+	+F		+++ HA	+
6	22		4		++M	++	++		+++ HA	+
7	16		2		—M	—	—F		+++ HA	—
8	14		2		—M	—	—F		+++ HA	—
9	14		4		—M	—	—F		+++ HA	++
10	14		4		—M	—	—F		++ HA	—
11		110	4		++M	++	++		+++ H	+++
12		110	4		++M	++	+++		+++ H	+
13		80	2		+M	+	++		+++ H	+
14		80	2		++M	++	+++		+++ H	++
15		130	4		+++M	+++	+++		+++ H	++
16		130	4		++M	++	++		+++ H	++
17		120	6		++M	++	++		+++ H	+++
18		120	5		++M	++	++		+++ H	++
19		100	4.5		++M	++	++		+++ H	+++
20	14			3	—L	—	F	—	h	—
21	4			0.5	—L	—	—	—	h	—
22	12			3	—L	—	—F	—	h	+
23	12			3	—L	—	—F	—	h	—
24		80		3	++L	+	+++	++	++ HB	++
25		60		3	++L	+	++	++	++ HB	+
26	20				neg.				Slight h	
27	20				neg.				Slight h	
28	22				neg.				Slight h	
29	22				neg.				Slight h	

F = Fibrosis.
A = Amaturation.
M = Myeloid.
L = Lymphoid.
B = Increase in erythropoiesis and eosinophils.
H = Hyperplasia.
h = hypoplasia.

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* Fellow of the Institute of International Education.

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myelokentric or crude lymphokentric acid. 0.5 ml to 1.0 ml of urinary extract was given daily in aqueous suspension at a pH of 7.8 for 9 to 14 days. The total dose of extract given was made from 2 to 6 liters of urine. Ten guinea pigs, as well as receiving crude myelokentric acid, also were given varying amounts of Aminopterin, the daily dose being 2 mg; and as controls 10 animals were given 10 mg daily of Teropterin as well as crude myelokentric acid. Crude lymphokentric acid was given to 6 animals; to 4 of them 2 mg daily doses of Aminopterin were given, and the other two were given 10 mg daily doses of Teropterin. Four animals were given 2 mg daily doses of Aminopterin only.

Results. The results are summarized in the table, in which it will be seen that animals that received crude myelokentric acid are designated under result as being positive for extra medullary myelopoiesis, + to +++M; or if negative for this finding, –M; and equivocal animals are marked ±. Liver, spleen and adrenals were examined for extra medullary myelopoiesis and are graded – to +++ under such headings. Animals that received crude lymphokentric acid were similarly graded as to the extent of lymphopoiesis in liver, spleen, lymph nodes, and adrenals and the results are tabulated for each animal as –L or ++L. Animals that received Aminopterin alone exhibited no myeloid or lymphoid stimulation, and these therefore are designated negative. The only change in this latter group is slight hypoplasia of the bone marrow.

It will be seen that animals 1 and 2 showed little effect of the drug on the extra medullary hematopoiesis. These 2 animals, however, were given only 2 doses of Aminopterin on the last two days of injection with urinary extract. All others that received Aminopterin and myelokentric acid, with the exception of 6, exhibited only minimal or no extra medullary blood formation. The bone marrows of this group showed hyperplasia, and in all except those from animals 1 and 2 there was some amaturation with little red cell formation. (Fig. 1). The spleens of all but 1, 2 and 6 were small and fibrotic.

All animals that were given Teropterin and myelokentric acid showed a fairly good response, *i.e.* extra medullary hematopoiesis in at least 3 organs. The bone marrows were hyperplastic in all elements. (Fig. 2)

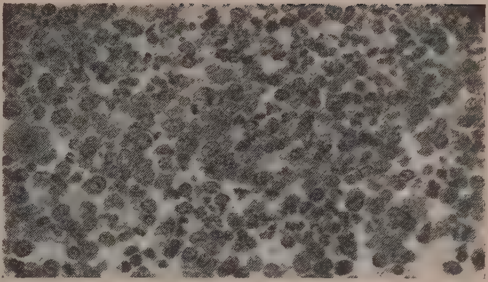

FIG. 1.
Section of bone marrow ×300, from animal that received crude myelokentric acid and Aminopterin showing hyperplasia with some amaturation of leukopoiesis. Erythropoiesis is less than normal.

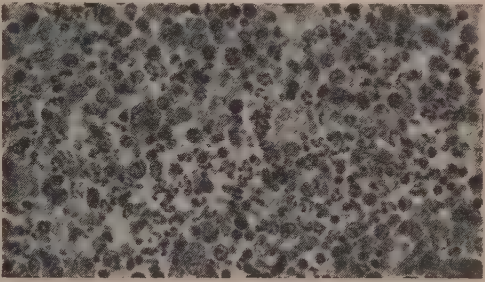

FIG. 2.
Section of bone marrow ×300, from animal that received crude myelokentric acid and Teropterin showing hyperplasia of all myeloid elements.

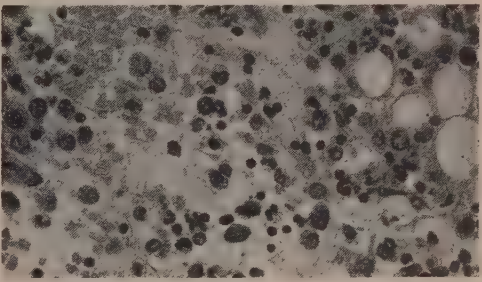

FIG. 3.
Section of bone marrow ×300, from animal that received crude lymphokentric acid and Aminopterin showing moderate hypoplasia of all myeloid elements.

The 4 animals that received Aminopterin and lymphokentric acid exhibited no lympho-

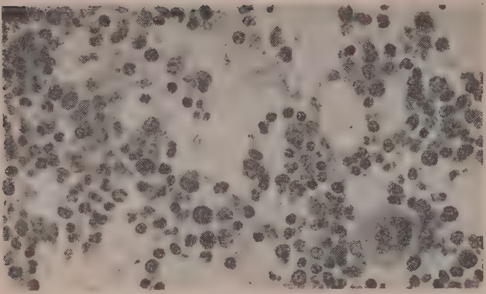

FIG. 4.

Section of bone marrow ×300, from animal that received Aminopterin showing mild hypoplasia of all myeloid elements, particularly the erythroid.

cytic stimulation. There was some fibrosis of the spleen in 20, 22, and 23, and all bone marrows were mildly hypoplastic and amaturative. (Fig. 3). The 2 control animals that received Teropterin and lymphokentric acid each showed a moderate increase in lymphopoiesis with, as well, an eosinophilia in the bone marrow and increased red cell production. Those animals that were given Aminopterin only exhibited mild hypoplasia of the bone marrow especially of the red cell elements. (Fig. 4). Otherwise these animals were negative. Fig. 1 to 4 represent the marrows from these four groups of animals.

Discussion. It would seem that Aminopterin interfered with the action of the lymphokentric acid more than that of myelokentric acid. This is evident in that the organs of animals 20, 21, 22, and 23 exhibited no increased lymphopoiesis; however, the bone marrows of these animals were more hypoplastic than those of animals that received Aminopterin only. In all animals the Aminopterin effected red cell formation more than white cell or platelet formation. It would be difficult to interpret the combined action of Aminopterin and these two acids, but the effects of Aminopterin on human leukemia are somewhat similar to the findings reported here.

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We wish to thank Mrs. Diva de Andino for technical aid in this work.

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Auto-Interference Associated with Influenza B Virus.*† (18242)

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The phenomenon of auto-interference is characterized by the presence, in concentrated viral inocula, of material which exerts an antagonistic or inhibitory effect on multiplication of the same virus. In the case of the influenza viruses serial passage of low dilutions of infected allantoic fluid in embryonated eggs was found to result in markedly lower infectivity and hemagglutinin titers than those obtained when high dilutions of the same inoculum were employed(1). Carefully controlled exposure of infected allantoic fluids to ultra-violet light was found to destroy or greatly reduce their infectivity, without gross destruction of the property of interference (2,3). The material responsible for the property of interference in such fluids has been shown to be intimately associated or identical with inactive virus(2,4).

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‡ It is a pleasure to thank Dr. Selman A. Waksman for encouragement throughout these studies.

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The experiments reported in this communication describe (a) an increase in the amount of interfering material present in allantoic fluid following death of the embryo, (b) the use of death of the embryo as a criterion for the measurement of infectivity and interference, and (c) the absence of demonstrable auto-interference following inoculation into the yolk sac.

Methods. The Lee strain of influenza B and the PR-8 strain of influenza A were used throughout these studies.§ Embryonated eggs were inoculated with 0.5 ml amounts into the yolk sac or allantoic sac on the 6th or 10th day of embryonic development respectively and the inoculated eggs were incubated at 36°C. Embryos dying during the first 24 or 48 hours after inoculation were routinely discarded. Hemagglutination tests were performed using the method described by Salk(5).

Experimental. It is well known that when influenza virus is propagated in the allantoic sac of embryonated eggs the maximum virus content is found in allantoic fluid harvested one to two days after inoculation from living embryos. Relatively few embryos die during the short incubation period and they are discarded. However, instances of embryo mortality following inoculation with the influenza viruses have been noted in the literature. Thus, death of the embryo was found to follow inoculation of influenza virus into the yolk sac(6,7) or allantoic sac(8-10) and embryo mortality following infection by the latter route was found to be greater when younger (9-day-old) embryos were employed (8). These observations were confirmed and extended in this laboratory.

TABLE I. Effect of Age of Embryo at Time of Allantoic Inoculation on Embryo Mortality.

Virus diluted	Influenza A virus		Influenza B virus	
	Embryos			
	9 day	11 day	9 day	11 day
10^{-0}	8/9*		9/9	10/10
10^{-1}	8/10	1/10	10/10	7/10
10^{-2}	6/6	1/10	9/9	8/9
10^{-3}	9/10	4/10	10/10	8/8
10^{-4}	6/8	3/10	10/10	7/8
10^{-5}	8/9	2/10	8/8	9/9

* No. embryos dead/total.

Data illustrating the effect of age of the embryo on mortality following inoculation of the influenza viruses into the allantoic sac are presented in Table I. It will be seen that (a) younger embryos succumbed more readily to infection with the influenza viruses than older embryos and (b) influenza B virus killed embryos with considerable regularity while influenza A virus did not. It will also be seen (Table IV, column L1) that embryo mortality and rate of embryo death following inoculation into the allantoic sac or yolk sac may be used as a criterion for the measurement of viral and anti-viral activity.

It is evident from the data presented in Tables I and IV that death of the embryo regularly follows intra-allantoic inoculation of influenza B virus. Since, in the case of infectious bronchitis virus of chickens, auto-interference was induced only by further incubation of infected eggs after death of the embryo(11), it was of interest to determine whether or not a similar phenomenon obtained with influenza B virus. Accordingly, eggs were inoculated with approximately 10^8 infectious units of influenza B virus by the allantoic sac route and pools of allantoic fluids from 10 or more eggs were collected from the following: *a*. embryos living 3 days after inoculation (L3), *b*. embryos dead on the third day after inoculation (D3), and *c*. embryos dead on the 3rd day after inoculation and held at 36°C for 24 hours after

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§ Obtained through the courtesy of Dr. Laurella McClelland.

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TABLE II. Mortality and Hemagglutinin Titer of Embryos Inoculated with Serial Dilutions of Various Allantoic Fluids.

	Inoculum: Allantoic fluid from eggs harvested					
	L3		D3		D3 + 1	
Inoculum dilution	D/T	Hem.	D/T	Hem.	D/T	Hem.
10^{-0}	10/10	>1280	10/10	1280	9/10	160
10^{-1}	9/10	>1280	10/10	320	3/9†	20
10^{-2}	9/10	>1280	3/10*	160	2/10*	160
10^{-3}	10/10	>1280	8/10*	>1280	7/8	>1280
10^{-4}	8/8	>1280	10/10	>1280	9/9	>1280
10^{-5}	10/10	>1280	10/10	>1280	9/10	>1280
LD_{50}	$10^{-7.4}$		$10^{-7.7}$		$10^{-6.1}$	
ID_{50}		$10^{-6.8}$		$10^{-7.5}$		$10^{-6.0}$

D/T = No. embryos dead/total.
Hem. = Reciprocal of hemagglutinin titer.
* All living embryos contained hemagglutinin in allantoic fluid.
† 4 of 6 living embryos contained hemagglutinin in allantoic fluid.
L3 = Embryo living on third day after inoculaton.
D3 = Embryo dead on third day after inoculation.
D3 + 1 = Embryo dead on third day after inoculation and held at 36°C for one day after death.

death of the embryo (D3 + 1). Serial decimal dilutions ranging from 10° (undiluted) through 10^{-5} of such fluids were inoculated into the allantoic sac of groups of 6 to 10 eggs each. Table II summarizes the results of 2 experiments. In the first experiment the various groups of infected embryos were candled daily for 6 days and the mortality ratios recorded. In the second experiment allantoic fluid was collected after 44 hours' incubation and the hemagglutinin titer determined on pools of the various fluids. It is evident from the data presented that little or no difference in embryo mortality or hemagglutinin titer was apparent among groups of embryos inoculated with various dilutions of allantoic fluid collected from living (L3) embryos. However, when serial dilutions of allantoic fluid collected from dead embryos (D3) were sub-inoculated, those embryos which received fluid diluted 10^{-2} had considerably lower mortality ratios and hemagglutinin titers than embryos receiving the same fluid undiluted or diluted 10^{-5}. This auto-interference was apparently enhanced when the inoculum was collected from dead eggs which were held an additional 24 hours at 36°C after death of the embryo (D3 + 1). The fact that auto-interference was less apparent in eggs inoculated with undiluted allantoic fluid than in the same fluid diluted 10^{-1} or 10^{-2} may be accounted for by the presence of excessive amounts of active virus. Thus, it would appear from the experiments described above that interfering material was liberated or produced following death of the embryo and that such auto-interference was masked by the presence of excessive amounts of active virus.

Exposure of infected allantoic fluid to heat (1,12) or ultraviolet light has been found to destroy or at least to drastically reduce infectivity of the influenza viruses without complete destruction of the property of interference(1,2). Furthermore, it has recently been shown that infectivity was completely destroyed by treatment with liquid ethylene oxide(13). It was of interest, therefore, to inactivate, by exposure to heat and to ethylene oxide, allantoic fluids collected from dead and living embryos and to determine the presence or absence of interfering material in such fluids. Accordingly, pools of various allantoic fluids were heated to 56°C for 40 minutes, 2 hours, 5 hours or 24 hours, respectively, and in one instance an aliquot portion was treated with 1% liquid ethylene oxide according to the method described by Ginsburg and Wilson(13). The inactivated fluids were inoculated into the allantoic sac, and thirty

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TABLE III. Protective Effect of Inactivated Allantoic Fluid on Embryos Infected with Influenza B Virus.

	First inoculation	Second inoculation	Result D/T	Result Hem.
Broth		Active virus*	26/28	1280
Allantoic fluid from				
D4	Heated at 56°C for 40 min.	Active virus*	1/26	20
D4	" " 56°C " 2 hr	" "	1/24	n.t.
D4	" " 56°C " 5 hr	" "	n.t.	40
K3	" " 56°C " 5 hr	" "	n.t.	40
D3 + 1	" " 56°C " 5 hr	" "	n.t.	20
D3 + 1	" " 56°C " 24 hr	" "	n.t.	320
D3 + 1	1% ethylene oxide for 1 hr at 10°C	" "	n.t.	80

* Allantoic fluid diluted 10^{-4}.
Hem. = Reciprocal of hemagglutinin titer.
n.t. = Not tested.

TABLE IV. Titration of Allantoic Fluids in Embryos by Different Routes.

Inoculum	Allantoic fluid from eggs harvested: L1				D3 + 1			
Route	Allantoic sac		Yolk sac		Allantoic sac		Yolk sac	
Inoculum diluted	D/T	A.D.D.	D/T	A.D.D.	D/T	A.D.D.	D/T	A.D.D.
10^{-0}	9/9	2.8	10/10	2.0	5/5	4.4	9/9	2.0
10^{-1}	9/9	3.2	10/10	3.1	5/10		8/8	3.0
10^{-2}	9/9	3.3	10/10	3.1	7/10		10/10	3.6
10^{-3}	10/10	3.8	9/9	3.7	9/9	4.0	9/9	4.0
10^{-4}	10/10	3.9	10/10	4.1	10/10	4.3	9/9	5.4
10^{-5}	10/10	4.5	10/10	4.6	8/9	4.9	7/10	
LD_{50}	$10^{-7.0}$		$10^{-7.1}$		$10^{-5.8}$		$10^{-5.9}$	

D/T = No. embryos dead/total.
A.D.D. = Avg day of deaths.

minutes later the same eggs, together with a control group previously inoculated with broth, were infected with approximately 10^3 infectious units of influenza B virus by the allantoic route: In one experiment the eggs were candled daily for 6 days after inoculation and in another experiment the hemagglutinin titers were determined on pools of 6 allantoic fluids. The results are summarized in Table III. It is clear that (a) the property of interference in allantoic fluids from either dead or living embryos was not destroyed by exposure to 56°C for 5 hours, (b) interference was strikingly demonstrated by embryo survival as well as by low hemagglutinin titer when allantoic fluid was inactivated with heat, and (c) the property of interference was not destroyed by exposure to 1% ethylene oxide for 1 hour at 10°C.

It has been shown in preceding sections that auto-interference occurred when low dilutions of unheated allantoic fluids from dead but not from living embryos were inoculated into eggs by the allantoic sac route. In order to determine whether a similar phenomenon obtained following inoculation into the yolk sac, allantoic fluid from both living and dead embryos was titrated in eggs by the yolk sac and allantoic sac routes respectively. The results are summarized in Table IV. It will be seen that when allantoic fluid from living embryos (L1) was titrated in eggs that (a) no evidence of auto-interference was found following inoculation by either route and (b) the rate of embryo deaths was dependent upon the amount of virus inoculated, irrespective of the route of inoculation. When allantoic fluid from dead embryos (D3 + 1) was titrated by the allantoic sac route, auto-interference, as indicated by embryo survival, was obtained following inoculation of the lower dilutions. However, when the same fluid was titrated by inoculation into the yolk sac no evidence whatever of interference was found. It is clear, therefore, that inoculation into the yolk sac was a suitable method for the measurement of infectivity but not for the demonstration of interference. In another experiment allantoic fluid from dead embryos was partly inactivated by exposure for 20 minutes at 56°C and titrated in eggs by both the yolk sac and the allantoic sac routes. As anticipated, mortality following inoculation into the allantoic sac varied from 0 to 30% in all groups. However, when the same fluid was titrated by the yolk sac route, the LD_{50} was $10^{-2.8}$, and embryos inoculated with undiluted fluid died more rapidly (A.D.D. = 3.2) than those receiving fluid diluted 10^{-1} (A.D.D. = 5.3). Thus it was not possible to demonstrate interference following inoculation of partly inactivated allantoic fluids known to contain interfering material. It is suggested that inoculation into the yolk sac may prove to be of value in the detection of residual active virus in inactivated preparations.

Discussion. It has been shown in the preceding sections that interfering material was liberated or produced following death of the embryo infected by the allantoic sac route with influenza B virus and that such material was not destroyed by exposure to a temperature of 56°C for 5 hours. It would seem reasonable to assume for the moment that the interfering material present in allantoic fluid from dead embryos differs quantitatively rather than qualitatively from like material known to be present in fluid from living embryos(1-3), although the possibility of existence of an unrelated inhibitory material should not be ignored. The fact that virus was found to elute from the allantoic membrane in dead but not living embryos following inoculation into the allantoic cavity(14) may account, in part, for the increase in the amount of interfering material present in unheated allantoic fluid following death of the embryo. It is of considerable interest that the presence of excessive active virus in untreated allantoic fluid from dead embryos was capable of overcoming the effectiveness of the interfering material present in the same fluid (Table II). Indeed, it is not clear whether inactivation of infective allantoic fluid actually creates interfering material or simply reveals the presence of such material by the removal of excessive infective virus, or both.

Summary. Experiments are described illustrating (a) the use of death of the embryo as a criterion for the measurement of infectivity and interference, (b) an increase in the amount of interfering material present in allantoic fluid following death of the embryo and (c) the absence of demonstrable autointerference following inoculation into the yolk sac.

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Cholesterol and Fatty Acid Synthesis in Biotin Deficient Rats. (18243)

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Elevation of the serum cholesterol level in man(1) and in the rabbit(2) has been observed in biotin deficiency. To determine whether or not this rise represents an increased production of cholesterol, the rate of cholesterol synthesis in biotin deficient rats was measured by means of deuterium oxide. Because livers from biotin deficient rats have a decreased rate of pyruvate oxidation(3) and because biotin is necessary for the reversible carboxylation of pyruvic acid to oxalacetic acid(4,5), it was of interest to determine in addition whether the rate of fatty acid synthesis in the rat is influenced by the interference of biotin deficiency with normal pyruvate metabolism.

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* National Research Council Fellow in the Medical Sciences (1948-50).

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2. Curran, G. L., unpublished observations.

Experimental. Animal feeding experiment. Five male weanling rats of the Wistar strain weighing about 30 g each were placed in separate metabolism cages. Three of the rats received 15 g a day each of the egg white diet recommended by Emerson(6). The two control rats received similar portions of a diet identical except for the egg white, which was autoclaved to destroy its avidin content. After three weeks the three rats on the unheated egg white diet showed evidence of biotin deficiency. At this time all the rats were injected with sufficient 99.8% D_2O to bring the heavy water content of their body fluids to 1.5%. For the next 11 days the rats received drinking water containing 2.5% D_2O. One of the biotin deficient rats died 2 days after injection of the heavy water; the remaining 2 rats in this group showed the alopecia, dermatitis, spectacle eye and neuromuscular imbalance characteristic of biotin deficiency by the end of the experimental period. After the body fluids had been enriched with heavy water for 11 days, the rats were sacrificed.

Isolation procedures. A sample of body water was distilled from each rat carcass and then the fatty acids and the cholesterol, as the digitonide, were isolated from each carcass and liver(7).

Isotope analyses. Deuterium analyses were carried out by a micromethod(8).

TABLE I. Cholesterol Synthesis in Biotin Deficient and Control Rats.

Rat No.	Atom % excess deuterium in body water, day 8*	Atom % excess deuterium in carcass cholesterol	Atom % excess deuterium in liver cholesterol	Deut. content in chol. of carcasses relative to that in body water†	Deut. content in chol. of livers relative to that in body water†
Control No. 1	.668	.213	.321	32	48
Control No. 2	.650	.183	.294	28	45
Biotin deficient No. 3	.721	.135	.264	19	37
Biotin deficient No. 4	.589	.168	.248	29	42

* Rat No. 4 died on 7th day of D_2O administration and deuterium values are for that day.

$$\dagger \frac{\text{Atom \% deuterium in cholesterol}}{\text{Atom\% deuterium in body water}} \times 100.$$

TABLE II. Fatty Acid Synthesis in Biotin Deficient and Control Rats.

Rat No.	Atom % excess deuterium in body water, day 8*	Atom % excess deuterium in carcass fatty acids	Atom % excess deuterium in liver fatty acids	Deut. content in fatty acids of carcasses relative to that in body water†	Deut. content in fatty acids of livers relative to that in body water†
Control No. 1	.668	.060	.102	9	15
Control No. 2	.650	.070	.102	11	15
Biotin deficient No. 3	.721	.084	.155	12	22
Biotin deficient No. 4	.589	.041	.105	7	17

* Rat No. 4 died on 7th day of D_2O administration and deuterium values are for that day.

$$\dagger \frac{\text{Atom \% deuterium in fatty acids}}{\text{Atom \% deuterium in body water}} \times 100.$$

Discussion. It will be seen from the last column in Table I that there is no significant difference in the rate of cholesterol synthesis in the biotin deficient rat as compared to the

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TABLE III. Body Weights and Weights of Carcass and Liver Fatty Acids from Biotin Deficient and Control Rats.

Rat No.	Body wt at termination of D_2O adm. in g	Wt gain or loss as % of body wt at start of D_2O administration	Wt of carcass fatty acids in g	Wt of liver fatty acids in g	Combined wt of liver and carcass fatty acids relative to body wt at termination of experimental period in %*
Control No. 1	150	+43%	3.76	.13	2.5
Control No. 2	165	+50%	5.41	.13	3.4
Biotin deficient No. 3	83	—10%	.71	.05	.9
Biotin deficient No. 4	95	—11%	1.84	.07	2.0

$$* \frac{\text{Wt of liver fatty acids} + \text{wt of carcass fatty acids}}{\text{Body wt at end of experimental period}} \times 100.$$

normal. Therefore the elevation of the serum cholesterol level in biotin deficiency cannot be explained on the basis of an increased rate of cholesterol production. The most likely explanation remaining is suggested by the data of Okey(9), who found that avidin prevents the accumulation of cholesterol in the liver by making biotin unavailable. Thus in biotin deficiency, even with a normal rate of cholesterol synthesis, the newly formed sterol cannot be deposited in the liver but rather enters the blood stream with a resultant hypercholesterolemia.

The deuterium contents of the fatty acids (Table II) show that the rate of fatty acid synthesis in biotin deficient rats is slightly increased. It is apparent that biotin deficiency, despite interference with the reversible carboxylation of pyruvate to oxalacetate, does not present a major obstacle to fatty acid synthesis in the intact rat. The last column of Table III gives the ratio of the combined weight of liver and carcass fatty acids relative to the body weight. It will be seen by comparing this column with the last column in Table II that the two biotin deficient rats with the smallest ratio of fatty acids to body weight have the highest rates of fatty acid synthesis. This finding is in agreement with the observations of Bernhard and Steinhauser (10) who found that the rate of fatty acid synthesis in fasting rats increased as the ratio of total weight of body fatty acids to body weight decreased. The increased rate of fatty acid synthesis found in biotin deficient rats is therefore a non-specific reaction to inanition rather than a characteristic of biotin deficiency.

Summary. The rates of cholesterol and fatty acid synthesis in normal and biotin deficient rats were determined by means of heavy water administration. In biotin deficient rats the rate of cholesterol synthesis was found to be normal and the rate of fatty acid synthesis slightly increased. It was shown that the increased rate of fatty acid synthesis is related to the inanition accompanying biotin deficiency and not to the deficiency itself.

The author is indebted to Mr. I. Sucher for the deuterium determinations and to Dr. D. Rittenberg for his kind interest and helpful suggestions.

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8. Rittenberg, D., unpublished.

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A Further Study of the Response of the Opossum Kidney, *Didelphys virginiana* to injury. (18244)

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In previous communications(1,2) consideration has been given to changes in the opossum kidney of an atypical order, resulting from an injury of an unknown nature, which resulted in the establishment of a cytological reaction on the part of these organs which would fall in the category of a chronic type of nephropathy very largely confined to the glomeruli. To the total number of 58 opossums previously studied, there must now be added observations on 5 additional animals. The present investigation is based not only on the study of the kidneys of the 5 new animals but on a critical review of the histological material representing the group as a whole; 63 adult animals of unknown age. In both of the above referred to studies as well as in this further study, the observation of dominant interest has been in the reaction of the glomeruli of 19 of these animals to an injurious agent. In the kidneys of such animals there occurs a variable number of glomeruli larger in diameter than unaffected structures in which the capsule is lined by a parietal layer of Bowman's membrane consisting of high, usually discrete, columnar epithelial cells. These cells decrease in height and the degree of cellular differentiation as the base of the glomeruli is reached. At this point there is a sharp transition of this order of columnar cell to the flattened, squamous type of cell normal for the glomerular membrane which is reflected as the visceral layer of epithelium over the capillary tufts. Such capillary networks are usually definitely abnormal in structure with thickened walls which frequently have the appearance of syncytial layers of endothelial tissue. Fig. 1 and 2.

In the previous studies(1,2) no explanation was offered for the development of these atypical glomeruli. A suggestion, however, was offered for the transition in cell type between the parietal and visceral layers of epithelium as occurs in such atypical structures. Such an explanation was found in the assumption that the flattening of the visceral

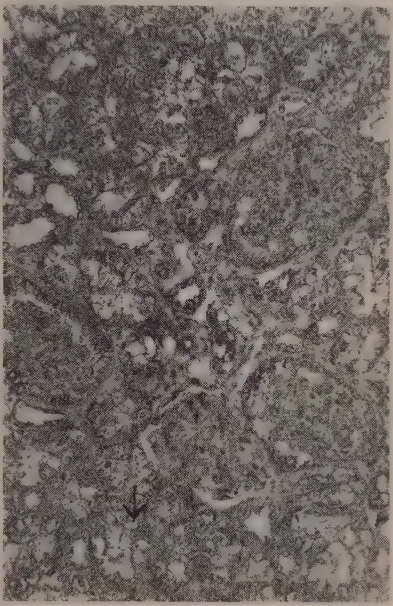

Fig. 1.

Microphotograph. Zeiss ×150, H & E. The figure is from the cortical area of an adult opossum kidney. Four glomeruli are included in the figure. The lower of these 4 structures is normal in size and cytological structure. Both the parietal and visceral layers of Bowman's membranes are formed by a flattened, squamous type of epithelial cell. The capillary tufts are distended and uniformly fill the space enclosed by Bowman's capsule. The upper 3 glomeruli in the figure are of larger size than the structure just described. In each one of them the parietal layer of Bowman's membrane if formed by cells which vary in height from a columnar order of cell to those of a flattened, squamous type. The flattening out process leading to a transition in cell form has not occurred uniformly over the surface of the parietal layer of the membrane. The capillaries of the glomerular tuft vary in their patency and degree of distension.

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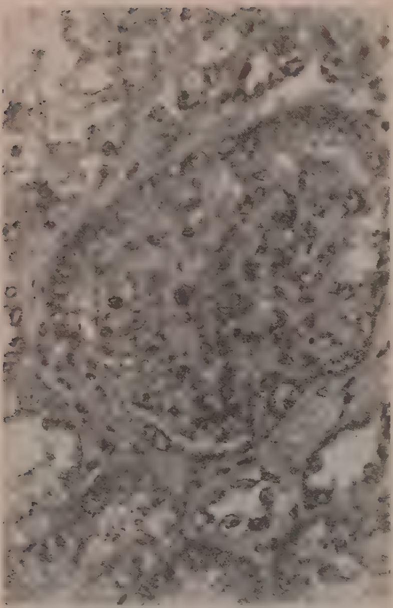

FIG. 2.
Microphotograph. Zeiss ×350., H & E. The figure shows one glomerulus under high magnification from the cortical area of the kidney. The cells of the parietal layer of Bowman's membranes though varying in height are of the columnar type of cell with pale nuclei and only in one area show evidence of the compression or flattening out process leading to the squamous order of cell. The capillary loops of the glomerulus are not discrete but appear fused, poorly differentiated and imperfectly distended with blood. Bowman's capsule is thickened and gives in general a hyaline appearance.

layer of cells was in part due to the pressure exerted on them with each ventricular systole as the capillary loops of such glomeruli became distended with blood and under pressure impinged upon and stretched the visceral layer finally resulting in a permanent flattening out of such cells. The delayed flattening of the columnar order of cell which constitutes the parietal layer of Bowman's membrane is likely due to the initial distension of the capillary loops having dissipated the force within these vessels before they fill the space encompassed by the glomerular capsule and impinge upon the atypical cells forming the parietal layer of Bowman's membrane. The explanation is strengthened by the observation that the columnar type of cell in the parietal layer of this membrane is not evenly or uniformly changed as it makes its transition to a flattened order of cell but is associated with the ability of capillary loops in a given area of the glomerulus to impinge and exert pressure upon a given section of the cells forming the parietal layer of the membrane. Those capillary loops showing endothelial thickening with partial or complete loop occlusion correspond to the areas on the parietal layer of Bowman's membrane which show the

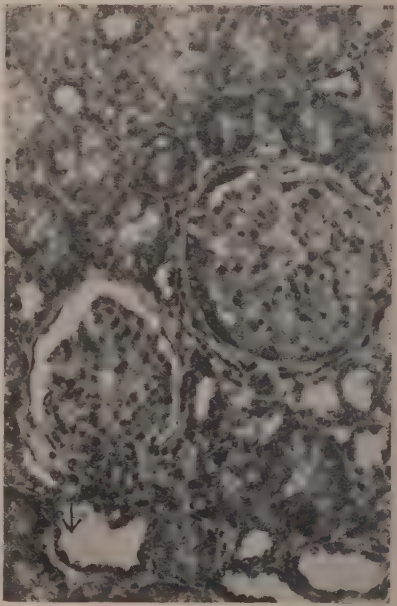

FIG. 3.
Microphotograph. Zeiss ×188, H & E. This figure from the cortex of the opossum kidney presents 2 glomeruli. The lower structure though increased in size is of normal cellular composition. The parietal layer of Bowman's membrane is uniformly formed by a normal order of flattened, squamous type of epithelium which when reflected over the glomerular capillaries maintains this same characteristic. The capillaries show imperfect lobulation and are not distended with blood. The upper glomerulus in this figure is greatly increased in size and appears distended by its capillary loops. In this structure are shown at various areas of the parietal layer of Bowman's membrane different types of epithelium in their transition from a columnar order of cell to the flattened type normal for the membrane. In those two areas in the glomerulus at which the capillaries fail to impinge upon the parietal layer of the membrane the cell type remains of a higher order in its configuration.

least change in cell type. Fig. 3.

In the kidneys of 2 of the 5 opossums studied in the present report and in 6 of those animals subjected to a critical review, structures have been observed, which with reservation may be designated atypical glomeruli in the process of formation. The existence of such atypical structures developing only in an injured kidney of an adult animal in a process of repair may in part explain the origin of those glomeruli which have been previously described as having an epithelial lining of an abnormal cellular design. The site of formation of these bodies has followed no definite pattern but have in general been more frequently observed in the corticomedullary area of the kidney. The development of such structures is brought about by one arterial vessel, variable in diameter, impinging against the wall of the renal tubule and invaginating this wall so as to form a double layered sac. The course of events is suggestive of the process involved in the usual embryological formation of the glomeruli. The walls of such an arterial vessel responsible for a given tubular invagination show the presence of smooth muscle cells. Such vessels are usually distended with blood and at the point of impact against the tubular wall undergo a sharp reduction in caliber. As the process of invagination progresses such vessels break into capillary structures containing blood cells. This vascular development would constitute the afferent element in the glomerular mechanism. In only 2 instances has it been possible to delineate clearly the efferent vessel arising from the capillary glomerular bed while at the same time maintaining in focus the afferent vessel. The circulation through such abnormal structures, comprising as they must a part of the general renal circulation has not been ascertained. For such an elucidation an appropriate injection technique would have to be employed preferably before the animal was sacrificed. The epithelial cells lining this two layered structure in the process of development by a process of invagination have not shown much difference in size or configuration. In general it would appear that the cells covering that part of the tubule which is in the process of becoming invaginated and therefore corresponding in a normal glomerular structure to its visceral layer, are of a lower and more flattened nature than similar cells on that portion of the tubule receiving the invagination. Fig. 4. The following measurements are from one of these atypical glomerular like bodies:*

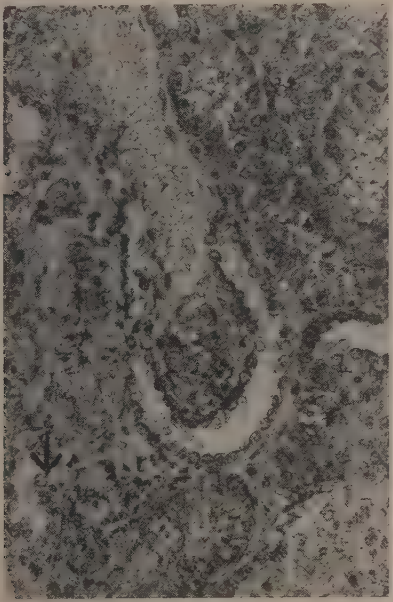

FIG. 4.

Microphotograph. Zeiss ×250, H & E. The figure shows a glomerular like structure from the cortex of the opossum kidney in the process of formation. A vessel, arterial in character, with smooth muscle cells in its wall has impinged upon a renal tubule and is in the process of inverting one wall of the tubule with the formation of a 2-layered sac. Capillaries in the process of formation are covered by what would correspond in the normal glomerulus to the visceral layer of Bowman's membrane. These structures as a whole are encompassed by the outer layer of the invaginated tubule which in turn would correspond to the parietal layer of Bowman's membrane. The cells in the outer layer of the invaginating structure, though poorly differentiated, appear in general to have a greater height than those cells forming the inner layer of the same structure.

The measurements included in the body of this paper were made from the atypical glomerulae like structure of Fig. 4.

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*I desire to thank Professor Edward C. Pliske, formerly of the Department of Anatomy of the University of North Carolina, for his courtesy and care in obtaining these values

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Long diameter of tubule bow	185.0
Short diameter of tubule bow	111.0
Long diameter of glomerular knot	148.0
Short diameter of glomerular knot	61.5
Arterioles of knot	7.5
Maximum epithelial height	9.0
Minimum epithelial height	4.5

Discussion. For a number of years research in this laboratory has very largely concerned itself with a study of the processes of epithelial repair in the kidney and the liver when these organs were effecting such a process as a result of either slight or severe mechanical or chemical injury(3-7). The observation has been made that the factor of tissue difficulty, strain, as these organs attempt their cytological restoration influences the morphological characteristics, the type of epithelial cell, which either transitorially or permanently establishes the process of repair. A slight injury is followed by the reappearance of a normal order of cell for the location in which the injury has occurred. A very severe injury registers its influence in the process of repair by a reversion to an embryonic order of epithelial tissue, frequently syncytial in nature and showing no or very poor cellular differentiation. In a certain number of instances such an order of repair remains over months or even years as a permanent morphological transition while in other instances there would appear to remain in this embryonic order of tissue of repair the urge to return to the differentiated type of cell normal for this area that has participated in the severe injury.

The injury which has been described for the opossum kidney shows itself in a dominant fashion, not in the tubular epithelium of this organ, but to a similar tissue (epithelium) found in the glomeruli. In a certain number of these ancestrally old animals, the marsupials, it would appear that the repair process may also revert to an embryonic status not only in the type of cell which is found in the two layers of Bowman's membrane but furthermore and rarely, this reversion may take on structural proportions with an attempt to form new bodies atypically glomerular in their configuration.

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Differential Effect of Desoxycorticosterone and the Sodium Hormone on Eosinophil Count in the Mouse. (18245)

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Certain steroids from the adrenal cortex cause a reduction in the number of circulating eosinophils. Seventeen-hydroxy-11-dehydrocorticosterone and related compounds are most potent. Desoxycorticosterone in large doses will cause eosinopenia(1). Meyer and Speirs(2) have recently modified the Thorn(3) test for the assay of the compounds. It occurred to us that this might be another means of comparing desoxycorticosterone and the sodium hormone. We have been able to show that the sodium hormone is without effect on the eosinophils, while we

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TABLE I. Effect of DC and Sodium Hormone on Eosinophil Cells in Mice.

Dose, γ	No. of determinations	Eosinophil count		Change, %
		4 hr	7 hr	
		Sodium hormone		
300	6	62	56	—10
380	6	50	54	+ 8
758	2	25	19	—24
1516	2	53	56	+ 6
		DC		
25	4	62	70	+13
80	4	56	43	—24
150	8	62	22	—65
200	2	31	12	—61

have confirmed the observation that desoxycorticosterone (DC) causes eosinopenia. This furnishes additional evidence that desoxycorticosterone is not the sodium hormone.

Methods. The sodium hormone* was prepared from beef adrenals and separated from the gluconeogenic hormones by chromatography according to the procedure of Thatcher and Hartman(4).

Two strains of mice were used for the test since they showed no difference in the response. These were the C-57 Bar Harbor mice and an albino strain obtained from an Indiana dealer. Because the eosinophil counts vary greatly from time to time depending on the condition of the animal, young animals of the same age, weight, diet, and cage conditions were used(1). Forty-eight hours after adrenalectomy 5 γ of epinephrine hydrochloride were injected subcutaneously to eliminate any response due to adrenal rests. The eosinophil count at the end of four hours was used for comparison with the count made three hours after injection of the substance to be tested. The test substance was injected subcutaneously at the end of the 4 hour period.

Results. The results shown in Table I are typical. Additional determinations were made with similar effects but different dosages. It will be noted that the sodium hormone had no significant effect even in large doses while DC was effective in sizeable doses (150 γ or more).

Discussion. It has been shown previously that sodium hormone and DC differ in their chemical properties(4). They both produce marked sodium retention when injected into normal dogs. The preparations of sodium hormone used in our experiments were recently tested for this property and proven potent.

Speirs and Meyer(1) found that DC in relatively large doses (150 γ) caused maximum eosinopenia. Cortisone is very potent, as little as 5 γ producing maximum eosinopenia (unpublished results in our laboratory).

We conclude from our experiments that the sodium hormone does not produce eosinopenia whereas DC, in comparatively large doses, does.

Summary. Sodium hormone potent in respect to its ability to produce sodium retention failed to produce eosinopenia in adrenalectomized mice while large doses of desoxycorticosterone did. This is further evidence that the two substances are not identical.

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* We wish to thank Dr. Oliver Kamm of Parke, Davis and Co. for the adrenal glands from which the fraction was prepared.

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E. coli Serotype D433: Occurrence in Intestinal and Respiratory Tracts, Cultural Characteristics, Pathogenicity, Sensitivity to Antibiotics.*

(18246)

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Although progress has been made in the knowledge of the causes of diarrheal disease of infants, recognized pathogenic agents are frequently not recovered. Recently Taylor, Powell and Wright(1), in a study of infantile diarrhea and vomiting, described a serotype of *E. coli* (D433) which was associated with several outbreaks and which was not encountered in 208 control cases. Taylor and her associates found serotype D433 to be identical with the organism described by Giles and Sangster(2); it belongs to a new group, namely, Kauffmann's Group 0111. Since it seemed that this serotype may be of etiological importance in infantile diarrhea, the following studies were undertaken. (1) An attempt was made to determine whether this particular serotype is present in sporadic cases of infantile diarrhea and in *E. coli* infections (pyelitis, peritonitis, meningitis, etc.) of infants, children and adults. (2) The growth characteristics of this serotype on selective culture media were determined in order to ascertain whether it resembles coliform bacteria, being more or less inhibited, or whether it is similar to certain enteric pathogens which are able to grow profusely. (3) Since it was shown(3) recently that salmonellae may be encountered in the upper respiratory tract of children suffering from gastoenteritis, an observation that suggests the possible air-borne transmission of enteric infection from such patients to other individuals, studies on the presence of serotype D433 in the upper respiratory tract of infants were undertaken. (4) The sensitivity of this serotype to various antibiotics was determined *in vitro*. (5) Aureomycin therapy was evaluated in 4 patients suffering from diarrheal disease associated with this microörganism. (6) The effects of ingestion of this organism were observed in 1 infant.

Material and Methods. Through the courtesy of Dr. Joyce Wright, London, England, 4 strains of *E. coli* and 4 antisera were received. Three strains represent serotype D433; the fourth strain, labelled Beta, represents another type, described by Giles, Sangster and Smith(4) and belongs to Kauffmann's Group 055. Antisera were prepared in rabbits and proved to be identical with the sera supplied by Dr. Wright. The sensitivity of the strains to various antibiotics was determined *in vitro,* using serial dilutions of the antibiotics in infusion broth as well as discs containing the antibiotics on seeded Endo agar. Aureomycin hydrochloride (intravenous) was made available through the courtesy of Dr. B. W. Carey of Lederle Laboratories; pure crystalline chloramphenicol (chloromycetin) was kindly supplied by Dr. J. P. Gray of Parke, Davis and Co., polymyxin B sulfate ("Aerosporin") by Dr. D. S. Searle of Burroughs Wellcome and Co., and terramycin hydrochloride by Dr. Elliott R. Weyer of Charles Pfizer and Co. The other antibiotics were procured on the open market. Antibiotic discs containing various amounts of penicillin, streptomycin, chloromycetin, terramycin, and bacitracin were made available by Difco Laboratories through the courtesy of Mr. H. W. Schoenlein. Tablets containing aureomycin were purchased.

Results. E. coli serotype D433 was encountered in 5 infants with non-epidemic diarrheal disease.† All strains were typical of *E. coli* and were agglutinated to full titer by

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* Aided, in part, by a grant of Lederle Laboratories Division, American Cyanamid Co.

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† Since this report was prepared, serotype D 433 was isolated from 3 additional cases and the Beta type from another 3 infants with sporadic diarrheal disease.

D433 antisera. Moreover, one of the strains was sent to Dr. Taylor, who corroborated its identity by means of agglutination and agglutinin-absorption tests. Serotype D433 was not present in 33 additional patients with diarrheal disease, either associated with parenteral infection or of undetermined origin. Neither was it found in cultures of feces and of material from nasopharynx and throat of 23 normal premature infants harboring *E. coli* during the first week of life. *E. coli* isolated from urine, peritoneal exudate, spinal fluid, ear drainage and blood from 28 patients suffering from pyelitis (15 cases) peritonitis (6 cases), meningitis (2 cases), otitis media (3 cases), and septicemia (2 cases) were not of this serological type.

The 5 patients who harbored serotype D433 ranged in age from 5 weeks to 3 months. No other pathogenic bacteria were found. The organism was recovered from the feces of all patients, from the nasopharynx of 2 out of 4 and from the throat of 1 out of 4 cases investigated. It is important to emphasize that 3 of the children had neither history or evidence of vomiting. The fact that *E. coli* D433 was found in the throat and nasopharynx of one of the patients who did not have vomiting extends the observations of Taylor and associates(1) who had recovered this serotype from the nasopharynx of infants with vomiting but had not investigated its possible presence in the throat. It is evident that vomiting *per se* is not the sole reason for the presence of this organism in the upper respiratory tract. These observations, together with the recovery of salmonellae(3) and *S. sonnei* (unpublished data) from the upper respiratory tract of infants and children with diarrheal disease, suggest the possibility that these patients may serve as source for air-borne transmission of enteric disease. Following treatment with aureomycin serotype D433 disappeared within a period of 3 to 4 days in 4 patients. In contrast, this organism persisted in the feces of the fifth patient who was treated with sulfadiazine and penicillin. Data on the *in vitro* effectiveness of aureomycin are presented below.

Serotype D433 may be the predominant, gram-negative, aerobic bacillus in the feces of an infected infant as evidenced by the fact that of the 20 isolated colonies of *E. coli* present on Endo agar seeded with feces of 3 of the patients, all belonged to serotype D433. In the remaining 2 cases *E. coli* D433 represented approximately 50% of the coliform organisms present. The cultures from the nasopharynx and throat were comprised of approximately 10% serotype D433 and 90% other coliforms.

Regarding the epidemiological aspects of the diarrheal disease of the 5 patients harboring serotype D433, it may be stated that 2 were twins and that 4 developed the disease at 2 hospitals and one at home. So far as we are aware, there was no outbreak of "epidemic diarrhea of the new-born" at either hospital. These patients, then, have to be considered as sporadic cases of diarrheal disease associated with the presence of serotype D433 in the feces and/or upper respiratory tract. In 2 patients the antibody response was determined, but agglutinins against serotype D433 were not found, 7 and 17 days, respectively, after the onset of the disease.

Thus far, *E. coli* serotype D433 has been incriminated as etiological agent of diarrheal disease solely on epidemiological grounds. Ingestion with the formula of approximately 100 million viable organisms of *E. coli* serotype D433 by a 2-months-old infant with multiple congenital defects, including the brain, who did not previously harbor this microörganism in the feces or respiratory tract, resulted within 24 hours in diarrhea and weight loss of 7 ounces. On the following day serotype D433 was demonstrated in small numbers in the throat and in large numbers in the feces, but not in the nasopharynx. Two days later this organism was present in very large numbers in the nasopharynx, throat and feces. The infant then was treated with terramycin (50 mg/kg/24 hr) which resulted in clinical improvement and the disappearance of this organism within 48 hours. Ingestion of a different type of *E. coli* recovered from the feces of this infant prior to its contact with serotype D433 did not cause diarrhea or weight loss.

Schaub(5), studying the cultural characteristics of paracolon bacilli, found that some (Group 1) are inhibited on selective culture media, whereas others (Groups 2, 3, and 4) are not. The latter groups resemble the pathogenic enteric organisms. It was deemed of interest, then, to determine whether serotype D433 of *E. coli* is inhibited on such culture media. The strains received from Dr. Wright as well as those isolated in this laboratory were seeded on (1) Endo agar, (2) SS agar and (3) desoxycholate citrate agar. Small inocula were used (1:10,000 dilution of an 18 hour broth culture). It was found that all strains were either completely or considerably inhibited on the two selective media as compared to Endo agar. That these selective culture media are inhibitory is also evident from the fact that *E. coli* serotype D433 was isolated from Endo agar 9 times, from SS agar only once, and from desoxycholate citrate agar only twice, although all media were seeded with all specimens in like manner. It is obvious that these selective culture media should not be used exclusively in the examination of material for the presence of serotype D433 and that this possibly pathogenic serotype does not differ from ordinary coliform bacilli in its growth characteristics on selective media.

It is of interest to know whether *E. coli* serotype D433 differs in its sensitivity to various antibiotics from *E. coli* strains tested previously by various investigators. To this end, the 4 strains received from England and the 5 strains isolated in this laboratory were tested for their sensitivity to the more important antibiotics and sulfadiazine. The results are summarized in Table I. It is interesting to note that all strains were inhibited by the newer antibiotics, aureomycin, chloromycetin, terramycin, and polymyxin B, in a concentration of 10 μg per ml. Streptomycin was distinctly less effective. It is important to emphasize that the strains which were inhibited by small concentrations of one antibiotic were not necessarily inhibited by small concentrations of another. Essentially identical results were obtained in tests employing antibiotic discs and tablets. Obviously, several antibiotics offer themselves for treatment of patients harboring this type of *E. coli*. Observations on the effects of one of them, namely, aureomycin, were referred to above. Favorable results were reported with chloromycetin by Rogers, Koegler, and Gerrard(6). Further investigations on the potential value of different antibiotics in such patients are in progress.

TABLE I. Growth Inhibitory Effects of Various Antibiotics and Sulfadiazine on Nine Strains of *E. coli* Serotype D 433.

Antibacterial agent conc./ml	No. of strains inhibited after 24 hr at 37°C
Streptomycin, 100 μg	3
25	3
10	1
Aureomycin, 10 μg	9
5	2
3	2
1	0
Chloromycetin, 10 μg	9
5	6
2.5	4
1	0
Polymyxin B, 10 μg	9
5	7
1	2
Terramycin, 10 μg	9
5	2
1	2
Bacitracin, 400 U	9
200	4
100	0
Penicillin, 500 U	0
Sulfadiazine, 12.5 mg	2

Summary and conclusions. A study of the cultural characteristics and sensitivity to antibiotics of *E. coli* serotype D433 and its occurrence in infants and in patients suffering from various *E. coli* infections revealed the following: (1) Serotype D433 was recovered from 5 sporadic cases of infantile diarrhea; it was present in the feces of all, in the nasopharynx of 2 and in the throat of 1 of these patients. The presence of this microörganism in the upper respiratory tract renders air-borne transmission, particularly in nurseries, a distinct possibility. (2) Serotype D433 was found neither in 33 additional patients suffering from diarrheal disease nor in 23 premature normal infants harboring *E. coli* during the first week of life, nor was it recovered from 28 patients suffering from various *E. coli* in-

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5. Schaub, I. G., *Bull. Johns Hopkins Hosp.*, 1948, v83, 367.

6. Rogers, K. B., Koegler, S. J., and Gerrard, J., *Brit. Med. J.*, 1949, v2, 1501.

fections of organs other than the intestinal tract. (3) Ingestion of serotype D433 of *E. coli* resulted in diarrhea and weight loss in a 2-months-old infant, whereas a similar exposure to the patient's own strain of *E. coli* failed to do so. Terramycin therapy was followed by clinical improvement and the disappearance of *E. coli* serotype D433. (4) Serotype D433 (4 strains received from England and 5 isolated in this laboratory) were completely or considerably inhibited on SS agar and desoxycholate citrate agar but grew well on Endo agar. Obviously, these selective culture media should not be used exclusively in examinations for the presence of this organism. (5) Serotype D433 (the abovementioned 9 strains) was found to be highly susceptible to aureomycin, chloromycetin, terramycin, and polymyxin B (10 μg per ml), but less so to streptomycin. None of the strains were susceptible to bacitracin (100 U per ml) and penicillin (500 U per ml). (6) Treatment with aureomycin of 4 patients resulted in prompt disappearance of serotype D433 and clinical improvement; the fifth patient was treated with sulfadiazine and penicillin and continued to excrete this microorganism in the feces.

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Effects of a Water-Miscible Multiple Vitamin Preparation on Tissue Cultures.* (18247)

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(Introduced by F. J. Stare)

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The present study deals with the effects of a commercial water-miscible multiple vitamin preparation, Vi-Penta, on the morphology of mouse fibroblasts and on the beat of mouse heart fragments grown in tissue culture. This investigation was undertaken because of the possible association of the feeding of water-miscible vitamin preparations to premature infants and the development of an ocular condition, retrolental fibroplasia, leading to blindness(4).

Materials and Methods. All experiments were carried out in roller tube cultures following the technique of Gey and Gey(1). The fibroblasts studied were derived either from mouse embryo skin fragments or from heart fragments of 1- to 3-day-old mice. The tissue fragments were placed in a row in glass culture tubes and then covered with a layer of chicken plasma. After the plasma had clotted, a supernatant fluid of the following composition was added: Tyrode's or Gey's solution, 0.6 ml, heated horse serum§, 0.2 ml and chicken embryo extract, 0.2 ml. The tubes were then stoppered and incubated at 37°C in a rotor. Test materials were incorporated into the supernatant by replacing 0.1 ml of the salt solution with 0.1 ml of the material to be tested. The supernatant was changed approximately twice a week. The cultures of heart fragments were examined periodically in order to determine the number of fragments beating.

Results. Morphologic Effect. After 4 days incubation of mouse embryo skin fragments, a striking morphologic difference was observed in the unstained, living cells exposed to a 1:1,000 dilution of Vi-Penta as compared to

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1. Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, v27, 45.

§ The horse serum was heated at 56°C for 3 hours to decrease the lysis of the plasma clot(2).

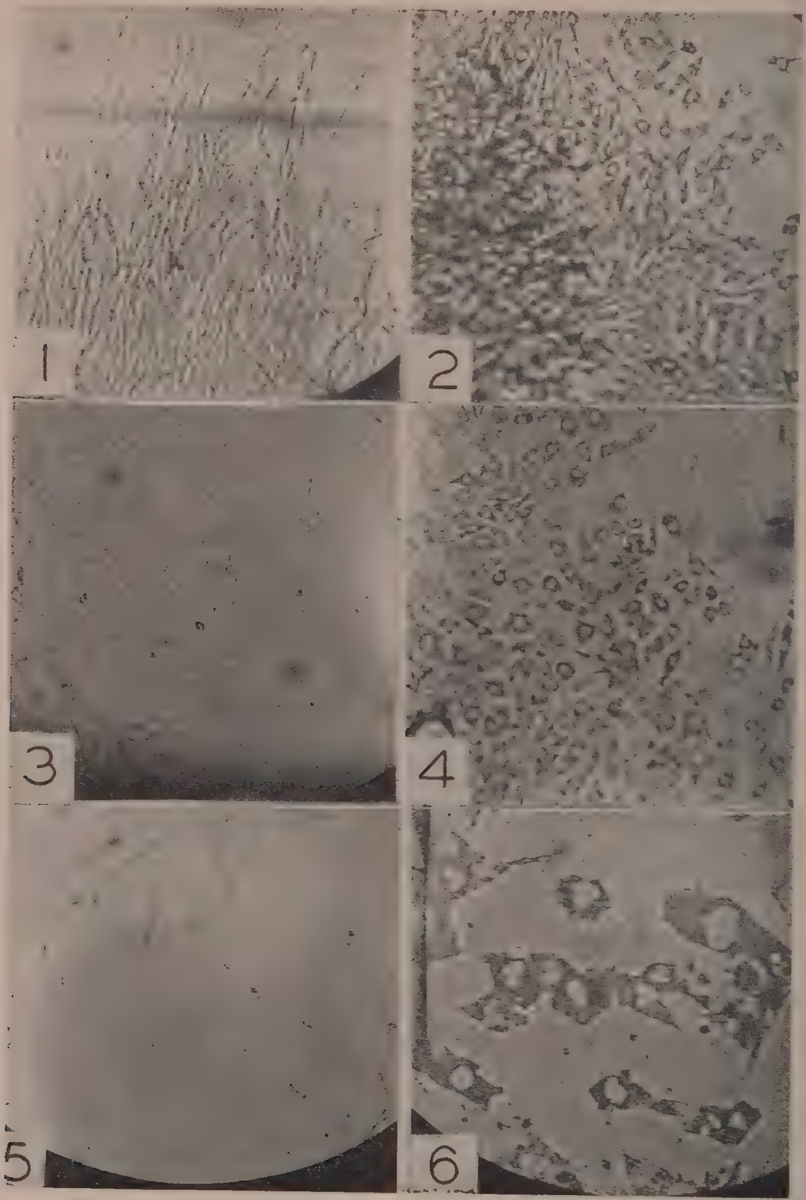

FIG. 1.

Part 1. Four-day growth of normal control cells from mouse embryo skin. (×60).

Part 2. Four-day growth of cells from mouse embryo skin which have been exposed to a 1:1,000 dilution of Vi-Penta. Note the broad, granular cells at the periphery of the outgrowth. (×60).

Part 3. Eight-day growth of normal control cells from mouse embryo skin. (×60).

Part 4. Eight-day growth of cells from mouse embryo skin which have been exposed to a 1:1,000 dilution of Vi-Penta. (×60).

Part 5. Fourteen-day growth of normal control cells from mouse embryo skin. (×120).

Part 6. Fourteen-day growth of cells from mouse embryo skin which have been exposed to a 1:1,000 dilution of Vi-Penta. Note the granularity of the cytoplasm, and the irregularity of the nuclear and cytoplasmic outlines. (×120).

TABLE I. Morphologic Effect of Various Dilutions and Fractions of Vi-Penta on Cells Derived from Mouse Embryo Skin and Heart Fragments.

Tissue	Substance tested	Dilution	No. of fragments	Presence of abnormal cells
Mouse embryo skin	Normal control	—	123	0
” ” ”	Vi-Penta	1:1000	93	Numerous
” ” ”	”	1:2000	28	Few
” ” ”	”	1:5000	18	0
” ” ”	” — vit. A & D fraction	1:1000	55	0
” ” ”	Vit. A & D fraction of Vi-Penta	1:1000	30	Numerous
Mouse heart	Normal control	—	115	0
” ”	Vi-Penta	1:1000	110	Numerous
” ”	Vi-Penta — vit. A & D fraction	1:1000	50	0

the normal control cells. Whereas the control cells maintained their normal thin, spindle-shape over the 14 days of culture, cells treated with Vi-Penta (especially those at the periphery of the outgrowth) showed an early broadening that in extreme cases amounted to a rounding with no extension of cytoplasmic processes (plate, parts 1 and 2). A more distinctive change in the treated cells was the notable increase in cytoplasmic granularity. While the control cells showed only the expected small amount of granularity in the cytoplasm near the poles of the nucleus, the treated cells appeared to have heavy granules filling their entire cytoplasm. This abnormal appearance of the treated cells was intensified after 8 days of incubation (plate, parts 3 and 4). By the fourteenth day many of the treated cells had undergone cytolysis, while most of the remaining cells had lost their processes and showed markedly irregular cytoplasmic outlines (plate, parts 5 and 6). During the early stages of the process, the nuclei of the treated cells appeared unaffected. By the fourteenth day, however, the regular, elliptical outline of the nucleus had become irregular, the nuclear material occasionally appearing to have been extruded into the cytoplasm.

The effects of various dilutions and fractions¶ of the vitamin preparation on the cell morphology are shown in the table. The data show that abnormal cells were produced by dilutions of the vitamin preparation as high as 1:2,000. In addition, the data also indicate that the responsible agent is associated with the vitamin A and D fraction of the vitamin preparation, since omission of the vitamin A and D fraction eliminated the appearance of abnormal cells and the use of this fraction alone produced abnormal cells.

Similar effects also were obtained with fibroblasts derived from newborn mouse heart fragments (table). However, tests with water-miscible solutions of either crystalline vitamin A or irradiated ergosterol (in concentrations approximating those in the Vi-Penta solutions) failed to produce the abnormal cells.

Physiologic Effect. It is well known that fragments of heart tissue grown in tissue culture will contract rhythmically for several days. As the cultures age, the number of pulsating fragments decreases progressively. Upon renewal of the supernatant, however, all the fragments usually resume their contractions. From the figure it may be seen that a marked decrease in the number of beating fragments occurred when Vi-Penta

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¶ Supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

2. Goldhaber, P., Cornman, I., and Ormsbee, R. A., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 590.

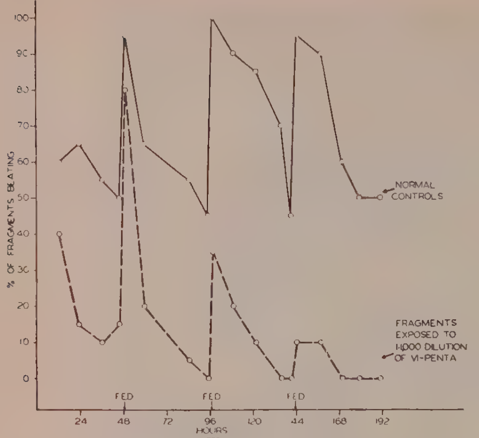

FIG. 2.

The effect of a 1:1,000 dilution of Vi-Penta on the number (in per cent) of beating heart fragments. Note the marked decrease in the number of beating fragments when Vi-Penta was present in the supernatant.

was present in the supernatant. Moreover, after renewal of the supernatant, these fragments did not completely resume their pulsations.

Discussion. The morphologic effects reported in the present experiments indicate that a 1:1,000 dilution of a commercial vitamin preparation, Vi-Penta, is toxic to mouse fibroblasts grown in tissue culture. While the responsible agent appears to be associated with the vitamin A and D fraction of the preparation, similar effects were not obtained with crystalline vitamin A or irradiated ergosterol.

Apparently, the morphologic effects observed were phenomena associated with a degenerative process. Evidence in support of this supposition was observed in the ability of the abnormal cells to stain with osmic acid, an indication of the lipoidal nature of their cytoplasmic granules, which Horning and Richardson(3) believe to be the predominant feature of the degenerating cell *in vitro*. In addition, the nuclear changes observed were similar to those found by Ludford(5) in the degenerating cell. It appears, however, that the toxic effects described are not specific for any particular agent, since similar abnormal cells have been observed in cultures not fed for a week, and also in cultures treated with high concentrations of the wetting agent‖ used in Vi-Penta.

With regard to the pulsating heart fragments, the most likely explanation for the normal, progressive decrease in the number of beating fragments with time is the exhaustion of some necessary component of the supernatant or the increased concentration of some metabolic waste product. The action of Vi-Penta, in markedly accelerating the normal decrease in the number of beating fragments and in preventing full recovery upon the addition of fresh supernatant, is an indication of its ability, at the concentrations used, to interfere with the normal metabolic processes of this tissue as well as cells. It should be noted, however, that similar effects were obtained with another commercial water-miscible vitamin preparation from a different source, thereby indicating that Vi-Penta is not unique in its action.

Summary. 1. The effects of a commercial water-miscible multiple vitamin preparation, Vi-Penta, on mouse fibroblasts and beating heart fragments grown in tissue culture were investigated. 2. Cultures exposed to a 1:1,000 dilution of the vitamin preparation for a period of 2 weeks were markedly affected morphologically. The most distinctive changes were a notable increase in cytoplasmic granularity and a broadening of the cells. 3. The toxic agent responsible for the morphologic effects appears to be associated with the vitamin A and D fraction of the preparation. However, crystalline vitamin A and irradiated ergosterol had no abnormal effect on the cells. 4. A marked and abnormal decrease in the number of beating heart fragments occurred when Vi-Penta was present in the supernatant. 5. Vi-Penta is not unique in its morphologic and physiologic effects, since tests with a com-

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3. Horning, E. S., and Richardson, K. C., *Australian J. Exp. Biol. Med. Sci.*, 1929, vVI, 229.

4. Kinsey, V. E., and Zacharias, L., *J. A. M. A.*, 1949, v139, 572.

5. Ludford, R. J., *Protoplasma*, 1935, v23, 180.

‖ The amount used was ten times the concentration of wetting agent in the 1:1,000 Vi-Penta solutions.

mercial water-miscible vitamin preparation from a different source gave similar abnormal effects.

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Comparative Bioautography of Vitamin B_{12} and Related Growth Factors Using *Euglena gracilis* and *Lactobacillus leichmannii*. (18248)

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It has been reported by Hutner *et al.*(1) that *Euglena gracilis* var. *bacillaris*, an algal flagellate, exhibits a quantitative growth response to crystalline vit. B_{12}, and that the desoxyriboside thymidine is inactive for this organism. Winsten and Eigen(2), and Cuthbertson and Smith(3,4) have used paper chromatographic and bioautographic procedures to demonstrate that certain *Lactobacillus leichmannii* strains exhibit a growth response to thymidine and other desoxyribosides in addition to vit. B_{12}. The procedures used by these authors demonstrated that the vit. B_{12} was found in the area of the slow-moving factors on the chromatograms while the desoxyribosides studied appeared as the more rapidly moving growth factors. Hutner's group(1) suggested that comparative studies using *E. gracilis* and lactobacilli would be of value in investigating the functions of vit. B_{12} and related growth factors. This communication presents a method for the use of *E. gracilis* as a bioautographic microorganism, and the application of paper chromatography and bioautography to the comparative study of the response of *E. gracilis* var. *bacillaris* and *L. leichmannii* 313 to vit. B_{12} and related growth factors.

Methods and materials. The general method employed in these comparative studies consisted of subjecting to paper chromatography selected samples of various preparations in duplicate, bioautographing one of the strips with *E. gracilis* var. *bacillaris* (ATCC 10616) and the other with *L. leichmannii* 313 (ATCC 7830) and finally comparing the positions of the areas of growth response of the two organisms. Descending chromatograms were prepared essentially as described by Winsten and Eigen(2) using *n*-butanol saturated with water as the solvent. Twenty-five μl of the material to be examined were applied to a 1 x 20 inch strip of Whatman No. 1 filter paper. After the desired distance of solvent flow had been attained, the strips were removed from the chamber and allowed to air dry in a disinfected hood.

The use of *E. gracilis* as a bioautograph organism required additional precautions against contamination because of its long incubation period. For this reason, aseptic technics, as far as practicable, were used. It appeared that *n*-butanol was in most cases an effective antiseptic agent, so that the wet chromatograms, if dried in a disinfected hood and thereafter handled aseptically, would remain essentially sterile. Large pyrex baking dishes with close fitting plate glass covers, used to contain the nutrient agar layers, were sterilized in an oven at 150°C. The medium was that of Hutner *et al.*(1) with 1.5% agar added, and was sterilized in the autoclave at

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2. Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, v181, 109.

3. Cuthbertson, W. F. J., and Smith, E. Lester, *Biochem. J.*, 1949, v44, v.

4. Smith, E. Lester, and Cuthbertson, W. F. J., *Biochem. J.*, 1949, v45, xii.

121°C for 15 minutes. The culture was maintained and the inocula were prepared as described by these authors. Subsequent operations were carried out in an inoculation room which had been disinfected with phenol and germicidal lamps. The medium was poured in two layers; a base layer, and a seed layer containing 10 ml of a 6- to 7-day-old culture of *E. gracilis* per 100 ml of medium. Since the organism is heat labile, it was necessary to cool the medium used for the seed layer to about 40°C before adding the inoculum, and to pour the medium onto the plate quickly. After solidification of the agar, the paper strips were laid down on the seeded agar surface, and removed in about 5 minutes. The plate was then incubated 4 days at room temperature in the inoculation room under four 18 inch "daylight" fluorescent lights placed about 18 inches above the plate. Visible response of *E. gracilis* to vit. B_{12} began to appear in 2 days, but 4 days of incubation were required for luxuriant growth. The growth response areas were well defined and a dense green, contrasting sharply with the remainder of the seeded agar plate which showed insignificant growth.

The bioautographs using *L. leichmannii* 313 were prepared as described by Winsten and Eigen(2). The medium used was that of Peeler *et al.*(5) with 1.5% agar and 0.025% sodium thioglycolate added. The growth response areas were clearly visible when viewed with oblique light against a dark background. The sensitivity of the bioautographic plates expressed as the least amount of crystalline vit. B_{12} that would give a definite visible growth when applied to the plate on a 1 cm^2 piece of filter paper, was determined. For *E. gracilis* this was approximately 0.01 mμg and for *L. leichmannii* approximately 0.5 mμg of vit. B_{12}. The results of the bioautographs of both organisms were recorded by direct photography and by full-scale line representation on graph paper. The strips illustrated in this paper were prepared by the latter method. The point of application of the sample and the final solvent front are shown on the strips.

In order to prove that strips prepared in parallel were actually comparable, or to confirm the existence or position of various activities, a split strip technic was used. In this procedure a single strip was chromatographed and cut lengthwise; one-half was applied to a *L. leichmannii* plate, and the other half to a *E. gracilis* plate. The quantitative tube method for interpreting chromatograms described by Yacowitz, *et al.*(6) adapted to single strips, was used with both of the test organisms to compare the growth response in liquid medium with that on the agar plates. Quantitative measurements of the activity of the various materials used were obtained by tube assay procedures using both organisms. The tube assay of Hutner *et al.*(1) using *E. gracilis* was modified by using 10 ml of medium, inoculating uniformly and aseptically with a pipetting machine, and reading color-turbidity at 420 mμ in a Coleman Junior Spectrophotometer. The method of Peeler, *et al.*(5) was used for the bacterial tube assay. The assay ranges for these 2 methods were found to be 0.01-0.15 mμg vit. B_{12} per 10 ml of medium for *E. gracilis* and 0.05-0.5 mμg for *L. leichmannii*. Several liver extracts, A.P.F. concentrates, and other materials of known or suspected activity were screened by these procedures. Of these, crystalline vit. B_{12} (Cobione, Merck), a 15 U.S.P. unit liver extract (Lederle), a sample of corn steep liquor (kindly supplied by Dr. L. A. Underkofler, Department of Chemistry, Iowa State College), and desoxyribonucleic acid (Nutritional Biochemicals Corp.) were selected for detailed study because of their representative and clearly defined patterns of activity. The first 3 were diluted with water to the desired concentration. Four preparations of desoxyribonucleic acid (DNA) were examined; (I) *DNA*—1 g of DNA was dissolved in 10 ml of dilute alkali. This prepara-

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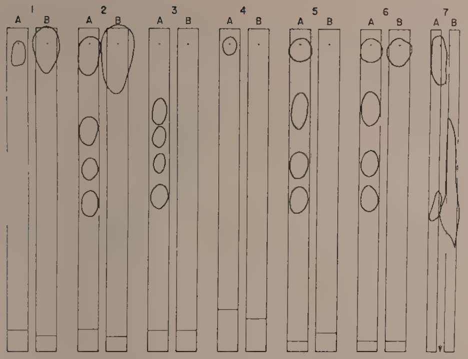

FIG. 1.

Comparative Bioautographs of Vit. B_{12} and Vit. B_{12} Active Preparations.

Strips 1A, 2A, 3A, etc.—*Lactobacillus leichmannii* 313. Strips 1B, 2B, 3B, etc.—*Euglena gracilis* var. *bacillaris*.

Twenty-five μl of the following preparations were applied to the corresponding strips. Vit. B_{12} and the apparent vit. B_{12} activities were measured with *L. leichmannii* 313.

Pair No.

1. Crystalline vit. B_{12} (Cobione); 10 $m\mu g$ per strip.
2. Liver extr., 25 $m\mu g$ as vit. B_{12} per strip.
3. Corn steep liquor, diluted 1:5, about 5 $m\mu g$ as vit. B_{12} per strip.
4. Desoxyribonucleic acid, about 1 $m\mu g$ as vit. B_{12} per strip.
5. Hydrolized desoxyribonucleic acid about 5 $m\mu g$ as vit. B_{12} per strip.
6. Same as (5) plus 10 $m\mu g$ crystalline vit. B_{12} per strip.
7. Same as (6), chromatographed 72 hours, a single strip cut lengthwise.

tion had some growth-promoting activity for *L. leichmannii*; (II) *Hydrolyzed DNA*—1 g of DNA was dissolved in dilute ammonia (final pH 8.5) and autoclaved at 121°C for 3 to 4 hours. The unhydrolyzed DNA was removed by precipitation with acid. This hydrolysis produced an activity *(L. leichmannii)* of 2 to 5 times that of preparation I; (III) *Norited DNA*—10 ml of preparation I were treated with Norite A for 30 minutes and filtered; (IV) *Hydrolyzed Norited DNA*—10 ml of preparation III were subjected to the same hydrolytic procedure described above. All of these preparations were adjusted to pH 5.0 before being applied to the paper strips.

The representations of comparative chromatographic bioautographs of these substances are shown in Fig. 1. The concentrations of the materials applied to the strips as designated in the legend were found in each case to give maximal growth while still retaining sufficient resolution. Since pure preparations of the vit. B_{12}'s and the desoxyribosides were not available, the criteria for evaluating the results of the bioautographs were the growth response to vit. B_{12} as found in Cobione, and the comparison of the R_F values obtained with those reported in the literature.

Results. Both organisms responded to crystalline vit. B_{12} (Fig. 1, Strips 1A and 1B). *E. gracilis* showed a larger area of growth response because of its greater sensitivity to vit. B_{12} and because of the greater diffusion of vit. B_{12} during the longer incubation period.

The response of *E. gracilis* to the liver extract (Strip 2B), differed markedly from that of *L. leichmannii,* showing growth only in the area of the slower moving factors where vit. B_{12} is expected to appear. Even when the concentration of liver extract was increased by 10 times, *E. gracilis* failed to show any response to the faster moving factors. This was confirmed by the chromatograph-tube method, which showed growth in only the first 2 tubes; that is, activity in only the first 2 inches of the strip. *L. leichmannii* on the other hand responded to 3 more rapidly moving factors in addition to the slow-moving factor (Strip 2A). The R_F value of the fastest moving factor indicated that it was thymidine(2,4), and the relative rates of the other moving factors generally correspond to those reported by Winsten and Eigen(2) as shown in Table I. So much liver extract had to be added to give well defined lower areas of growth that the double zone of the slow-moving activity was obscured. The use of less liver extract has shown a definite indication of a double zone of slow-moving activity.

E. gracilis showed no growth response to the chromatographed sample of the corn steep liquor (Strip 3B), while with an identical sample the *L. leichmannii* indicated the existence of 4 moving factors (Strip 3A), the R_F values and relative rates of which paralleled those reported by Winsten and Eigen(2) for their sample of corn steep liquor. Con-

TABLE I. Average Relative Rates and R_F Values for Vit. B_{12} Substitute Growth Factors (Test organism: *Lactobacillus leichmannii* 313)

	Moving vit. B_{12} substitute growth factors					
	1	2	3	4	5	5
Preparation	Relative rates* (2) (terms of Factor 5)					R_F† (Factor 5)
Liver extr.		.51		.78	1	.53
Corn steep liquor		.49	.60	.79	1	.52
Hydrolyzed DNA		.46		.77	1	.48
Values of Winsten & Eigen (2)	.32	.53	.63	.78	1	.52-.67

* Relative rate =

$$\frac{\text{Distance from starting point to center of growth zone in question}}{\text{Distance from starting point to center of fastest moving growth zone}}$$

† R_F value =

$$\frac{\text{Distance from starting point to center of growth zone}}{\text{Distance from starting point to solvent front}}$$

firmation of these results for both organisms was made with chromatograph-tube studies and direct tube assay.

E. gracilis showed no growth response to the chromatograms of any of the DNA preparations, two of which are illustrated by Strips 4B and 5B. Whenever vit. B_{12} was added prior to solvent flow to a strip containing any of the 4 DNA preparations, growth response with *E. gracilis* occurred, always in the area of slow-moving factors as shown on Strip 6B. The chromatograph-tube method and direct tube assay also indicated that *E. gracilis* was not stimulated by any growth factors in the DNA preparations. *L. leichmannii* demonstrated the existence of a slow-moving factor or factors present in the untreated DNA solutions, (Strip 4A). When this DNA solution was subjected to hydrolysis, the *L. leichmannii* bioautograph (Strip 5A) demonstrated an increased amount of growth in the area of slow-moving factors and the production of 3 rapidly moving growth factors. The *L. leichmannii* bioautographs of the chromatograms of norited DNA (not shown) demonstrated no areas of growth. This norite treatment actually removed very little of the DNA. Hydrolysis of this norited DNA solution resulted in a *L. leichmannii* bioautograph (not shown) which was practically identical with Strip 5A. The slow-moving factors produced by the hydrolysis may or may not have been identical with the slow-moving factors originally present in the DNA solution. The rapidly moving factors, as judged by their R_F values, correspond roughly to the 3 rapidly moving factors found in liver extract. A fourth moving factor, reported by Winsten and Eigen(2), was either not obtained by this type of hydrolysis, or was not resolved on the paper. The addition of vit. B_{12} to the strip prior to chromatography did not change the pattern of the response of *L. leichmannii* to the DNA hydrolyzates, as shown by Strip 6A. Since the slow-moving factors, either originally present or hydrolytically produced in these DNA preparations, were growth promoting for *L. leichmannii* but not for *E. gracilis,* none of these factors can be considered as being identical with crystalline vit. B_{12}.

Further evidence that the slow-moving factors of the DNA preparations and crystalline vit. B_{12} are not identical was obtained by the separation of vit. B_{12} from these slow-moving factors. This separation was accomplished by a long-term (72 hour) solvent flow procedure, the solvent being allowed to drip off the bottom of the strip. A split strip bioautograph of such a chromatograph is shown as Pair 7A and 7B. The more rapidly moving activities moved completely off the paper. The vit. B_{12} moved down a considerable distance, leaving a long trail that was picked up only by the more sensitive *E. gracilis.* The DNA factors remained practically stationary, and again did not promote the growth of *E. gracilis.*

Discussion. The use of *L. leichmannii* alone in the bioautographic examination of chromatograms of vit. B_{12} and vit. B_{12} substitute growth factors has led to the separation and identification of certain desoxyribosides as growth factors for that organism, but has not been particularly informative in the study of the slow-moving factors. These slow-moving growth factors may include not only vit. B_{12}, B_{12a}(8) and B_{12b}(9); but also nucleic acids, nucleotides(10), and other substances relatively insoluble in the *n*-butanol, that may serve as vit. B_{12} substitute growth factors for the test microorganism. Some resolution of these factors has been accomplished by altering the conditions of chromatography, such as using different solvents(4), treating the paper strips with salts (9), and extending the period of solvent flow.

That the comparative bioautograph technic using *E. gracilis* and *L. leichmannii* as described above gives further insight into the complex nature of the slow-moving group of factors is evident. *E. gracilis* showed a growth response to the vit. B_{12} of Cobione, but did not respond to the one or more unidentified slow-moving factors found in the DNA preparations which were highly growth-promoting for *L. leichmannii*. Additional work is needed, however, on the response of *E. gracilis* to vit. B_{12a} and B_{12b} before further information on the identity of the slower-moving factors can be ascertained by this procedure. Additional evidence that *E. gracilis* does not respond to many of the growth factors that are known to be active for the *L. leichmannii* strains, is apparent in all of the chromatograms presented in Fig. 1. Its lack of response to any of the rapidly moving factors substantiates and supplements the work and suggestion of Hutner *et al.*(1). The comparative bioautograph method may thus serve as a useful tool in the study of the slow-moving factors, as a qualitative method of indicating activities present in preparations which are to be assayed for vit. B_{12}, and as a guide in separation and purification procedures.

E. gracilis as a tube assay organism, although satisfactory for the relatively pure materials used in this investigation, in our hands has not given the desired results in the assay of complex natural materials such as animal tissues and blood.

Summary. 1. A method for the use of *Euglena gracilis* var. *bacillaris* as a bioautograph organism for the paper chromatographic study of vit. B_{12} and related growth factors has been described. 2. Evidence has been presented to indicate the value of comparative bioautographs, using *E. gracilis* and *Lactobacillus leichmannii* 313, in the study of these growth factors.

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A Simple Device For the Growth of Anaerobic Organisms in Liquid Media. (18249)

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The device described herewith (Fig. 1) is essentially a modification of the Theobald Smith fermentation tube(1). It consists of 2 test tubes differing in size which are fitted at the top with screw caps, and which are joined at the bottom with thick-walled capillary tubing. The plastic cap of the small tube is perforated with a drill-hole 2 mm in diameter, and both caps are supplied with solid heavy rubber gaskets so that air-tight seals are formed when they are screwed in place.

For the growth of anaerobic bacteria, the tubes are prepared in the following way: broth of the desired composition (tryptose phosphate; tryptisoy or a fresh meat infusion-

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1. Smith, Theobald. The Fermentation Tube with Special Reference to Anaerobiasis and Gas Production Among Bacteria, Wilder Quarter Century Book, Comstock Publishing Co., Ithaca, N. Y., 1893.

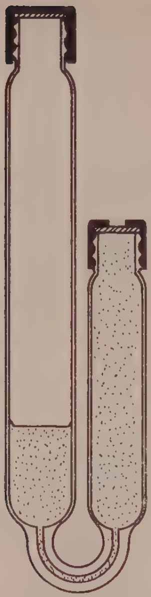

FIG. 1.
Anaerobic culture tube.

peptone broth containing a small amount of glucose) is added to fill the small arm. It is prevented from running into the large arm by tightening the cap on the latter. After the small arm is filled, its cap is screwed tightly in place, the cap in the large arm is removed and about 1 inch of broth is added to the large arm. The cap is replaced loosely on the large arm and the device with its contained broth is sterilized in an upright position in the autoclave by exposure to 121°C for 20 minutes. Pressure is then released slowly in the autoclave by shutting off the steam supply. When the tubes are taken from the autoclave and allowed to cool, the small arm will be found to be filled with broth. If methylene blue has been incorporated in the medium, the small tube will be seen to be in a reduced state, as shown by decoloration of the dye, whereas the medium in the large arm is oxidized, or becomes oxidized shortly from contact with the air which enters the arm. The reduced state in the small arm is maintained indefinitely (at least a month), whether the tubes are stored at room temperature or in the refrigerator.

The tubes are inoculated in one of 2 ways, depending on the quantity of inoculum available. When a large inoculum is used (*e.g.* approximately 0.1 ml of heavy broth culture), it may be added to the broth in the large arm by means of a sterile pipette. It is advantageous to deliver the inoculum at the orifice of the capillary and in many instances the cloudy inoculum can be seen to fall into the capillary. When a small inoculum must be used, it should be delivered into the small arm through the rubber gasket by means of sterile syringe and needle, care being taken not to introduce any air. Table I shows the smallest inoculum in terms of ml of original broth culture capable of initiating growth when introduced into the large arm by pipette, into the small arm by syringe and needle, and, for purposes of comparison, when grown in Brewer's thioglycollate medium.

Growth of the anaerobic organisms begins in the small arm, but when growth is particularly vigorous the medium in both arms will show growth and will be reduced as a result. Theobald Smith recognized the usefulness of his fermentation tube for the growth of anaerobic bacteria(1). The failure of his tube to come into general use for the purpose may be attributed to the inaccessibility of the anaerobic arm for inoculation or withdrawal of material, either of which can be done easily in the case of the modified tube. By means of this device, and larger models of it, any reasonable amount of anaerobic culture may be obtained in broth to which no agar, pieces of tissue or chemical reducing agents have been added. The tubes may be incubated in an ordinary incubator or water bath without the necessity of using an anaerobic jar, thus avoiding the disadvantage the latter has of exposing all its contained tubes to the air when a single tube must be withdrawn for study. Anaerobic bacteria which have been grown in the new device may be recovered from the broth culture by centrifugation, without sediment of tissue or agar to interfere. Furthermore, since the gas which forms during bacterial growth accumulates in the top of the small arm, to which access may be gained through the drill-hole and

TABLE I. Smallest Inoculum Required to Initiate Growth of Anaerobes.

Organism	Anaerobic device: Inoc. in large arm	Anaerobic device: Inoc. in small arm	Brewer's thioglycollate medium
Cl. perfringens	10^{-6}	10^{-9}	10^{-8}
Cl. lentoputrescens	10^{-6}	10^{-10}	10^{-10}
Cl. butyricum	10^{-4}	10^{-6}	10^{-7}
Cl. sporogenes	10^{-6}	10^{-8}	10^{-9}
Cl. tetani	10^{-1}	10^{-6}	10^{-6}
Cl. novyi	10^{-1}	10^{-3}	10^{-3}
Cl. bifermentans	10^{-1}	10^{-9}	10^{-9}
Cl. histolyticum	10^{-5}	10^{-11}	10^{-7}
Cl. septicum	10^{-4}	10^{-5}	10^{-6}
Cl. botulinum	10^{-4}	10^{-11}	10^{-8}

Serial dilutions of a heavy broth culture of the strains were made in 0.85% NaCl solution, so that the indicated inocula, in terms of original broth culture, were contained in 0.1 ml. The anaerobic device contained approximately 20 ml of broth of which 15 ml were in the small arm. Ten ml of Brewer's medium were used for testing. The end-point was the smallest inoculum which produced visible growth on incubation.

rubber gasket, the gas may readily be removed by syringe and needle or other apparatus for analysis.

Motile variants of bacterial organisms may be isolated from mixed cultures by means of the device. The tubes are filled with semisolid medium and an inoculum is placed near the surface of one arm. During incubation the motile variants grow down the inoculated arm, through the capillary and up the opposite arm to the surface, where they may be recovered readily.

The anaerobic culture device can be made by a skillful glassblower by using Kimble Neutraglass culture tubes with plastic caps (No. 45066) and Kimble Neutraglass capillary tubing (N-51A, No. 46486), outside diameter 7 to 8 mm. The gaskets can be punched from rubber sheeting (heavy inner tubing will do) by means of a cork borer of appropriate size.

Summary. A simple device is described by means of which anaerobic bacteria can be grown in liquid medium without the addition of chemical reducing agents or pieces of tissue, and without the use of an anaerobic jar or similar apparatus.

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Response to Adrenocorticotrophic Hormone in Clinical Scurvy.* (18250)

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A close relationship between ascorbic acid metabolism and adrenal cortical function is suggested by evidence derived chiefly from animal experiments(1-3). Diminished adrenal cortical activity might be expected, therefore, in human ascorbic acid deficiency. Accordingly, the change of the blood eosinophils and urinary uric acid following test doses of adrenocorticotrophic hormone (ACTH) was measured in 5 patients with clinical and laboratory evidence of scurvy before and after the administration of therapeutic amounts of ascorbic acid. Serum sodium and potassium concentrations likewise were measured before and after the specific antiscorbutic therapy.

Each of the 5 patients had typical scurvy as evidenced by perifollicular hemorrhages, ecchymoses, especially of the posterior thighs and buttocks, a positive tourniquet test, and

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* The expenses of this investigation were defrayed in part by a grant from Sharp & Dohme, Inc., Glenolden, Pa. to Harvard University.

† U. S. Public Health Service Postdoctorate Research Fellow.

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TABLE I. Effect of Test Doses of ACTH in Clinical Scurvy Before and After Ascorbic Acid Therapy.

Patient	W.W.	F.O'D.	J.C.	W.C.	P.C.
Age	66	74	67	36	75
Days between tests	22	20	12	*	7
Duration ascorbic acid therapy before 2nd test (days)	11	11	7	0	2
Total dose ascorbic acid (g)	13	11	7	0	6
Ascorbic acid content of buffy coat (mg/100 cc)					
1st test	0	4	3.4	0	0
2nd " ‡	37	23.7	10	—	0
4-hr eosinophil response† (cells/cu mm)					
1st test	115/35	0/0	210/67	57/4	150/31
2nd " ‡	64/10	17/10	289/84	—	144/47
Uric acid excretion† (mg/4 hr)					
1st test	84/49	—	86/44	14/17	143/66
2nd " ‡	65/10	60/43	40/78	—	131/65
Blood glutathione (mg/100 cc)					
1st test	25	20	35	26	12
2nd " ‡	22	21	—	—	15
Blood pressure (mm Hg)					
1st test	95/50	120/70	150/95	138/80	130/70
2nd " ‡	100/55	100/55	150/95	—	120/60
Serum sodium (mEq/L)					
1st test	140.3	129.4	138.5	147.5	—
2nd " ‡	138.5	134.7	143.5	—	—
Serum potassium (mEq/L)					
1st test	4.8	4.8	4.5	—	—
2nd " ‡	4.1	4.3	4.2	—	—

* Expired after 6 days of study.
† Tests before/4 hr after ACTH administration.
‡ After ascorbic acid.

a low buffy coat ascorbic acid content. Blood coagulation studies, including platelet counts, were normal.‡ Four of the patients gave dietary histories of prolonged ascorbic acid deficiency. The other patient (J.C.) had eaten tomatoes at irregular intervals. He also had a urinary tract infection. The interrelationship of the dietary and disease factors in this case is not clear, nevertheless clinical scurvy was present. All patients were examined within a few hours after hospital admission and were immediately transferred to the Thorndike metabolic ward. None of the patients was given ascorbic acid as such or in food prior to the study. During the period of study they were offered a diet consisting of boiled rice and milk, crackers, sugar, and coffee. This diet contained minimal quantities of ascorbic acid. One vial of ACTH§ was used for each test. The hormone was dissolved in saline and administered intramuscularly. Since other metabolic studies were concurrently conducted, the patients were not given ascorbic acid for from 5 to 11 days after admission. An ACTH test was done in all patients in the scorbutic state and repeated in 4 of these after the intravenous or oral administration of from 6 to 13 g of ascorbic acid. One patient expired on the sixth day of the study. Permission for autopsy was not obtainable.

Buffy coat ascorbic acid concentration was measured by the method of Butler and Cushman, which utilizes the reduction of 2-6 dichlorophenolindophenol(4). Urine uric acid was determined by the method described by Benedict and Franke(5). The blood eosinophil and urine uric acid investigations were conducted as outlined by Thorn, Forsham,

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‡ Carried out through the kindness of Dr. Frederick S Bigelow.

§ Provided by Dr. John R. Mote, The Armour Laboratories, Chicago 9, Ill. (Assayed to contain 35 mg of activity per vial compared to the standard).

4. Butler, A., and Cushman, M., *J. Clin. Invest.*, 1940, v19, 459.

5. Benedict, S. R., and Franke, E., *J. Biol. Chem.*, 1922, v52, 387.

Prunty, and Hills(6). Blood glutathione was determined according to the method of Woodward and Fry(7). Serum sodium and potassium concentrations were analyzed by flame photometry.

Results. The essential data are presented in Table I. In all patients prior to therapy, buffy coat ascorbic acid was absent (minimal dye reduction, probably of no significance, was observed in 2 instances). Following the administration of ascorbic acid 2 patients showed a return of the buffy coat ascorbic acid to normal and a definite but less marked increase occurred in one patient. Another patient (P.C.) who had been given less ascorbic acid over a shorter period of time than the others, showed no change. The reason for this is not evident since 4 g of ascorbic acid are usually enough to restore the ascorbic acid content of the buffy coat of a scorbutic patient to or near to normal(8). Following the administration of ascorbic acid, the tourniquet test was negative and ecchymoses were diminishing in all patients, including P. C.

The eosinophil response to ACTH in each instance was as great or greater than that considered indicative of normal adrenal activity, except for patient F. O'D. This patient had no circulating eosinophils prior to ascorbic acid administration. The administration of ascorbic acid did not alter significantly the control eosinophil counts nor the responses to ACTH in any of the patients studied.

An increase in the urinary excretion of uric acid after ACTH occurred in only one instance, in fact a decrease was most often observed. This paradoxical response is probably related to the low urine outputs which occurred in the 4 hours after the hormone, despite the ingestion of water. The ACTH possibly contained antidiuretic activity sufficient to account for this.¶

The blood glutathione concentration was either normal (25 to 35 mg per 100 cc) or slightly below normal in all instances in which the determination was done, except one patient (P.C.) whose concentration was low. There was no significant change in glutathione concentration following ascorbic acid administration. Since blood glutathione is largely confined to the red cells, alterations in red cell mass might be expected to influence whole blood glutathione concentration. Although hematocrits were below normal in 4 of the 5 patients, there was no correlation with the glutathione concentration, either before or after ascorbic acid administration.

Serum sodium concentration was normal in 3 patients in whom the determination was done, and low in one patient (F. O'D.). The administration of ascorbic acid did not appreciably alter these values. Normal serum potassium concentrations were found in the 3 patients in whom it was measured before and after ascorbic acid therapy.

Discussion. Four patients with clinical and laboratory evidence typical of scurvy had normal eosinophil responses to ACTH. This evidence of normal adrenal cortical function is further supported by the finding of initially normal eosinophil counts in these 4 patients and by the absence of eosinophils in the fifth patient with florid scurvy. Diminished adrenal cortical activity, as in Addison's disease, is accompanied by a high eosinophil count, a reduced serum sodium, an elevated serum potassium, and hypotension. In addition to normal eosinophil responses to ACTH, 3 of these patients with scurvy had normal serum sodium and 3 normal potassium concentrations prior to ascorbic acid therapy. Hypotension was not a prominent or consistent finding in these patients. The urine uric acid excretion after ACTH was not interpretable, probably because of the low urinary outputs after hormone administration. According to present concepts, the normal eosinophil response to ACTH and the failure to find consistent hypotension, hyponatremia, and hyperkaliemia, indicate

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6. Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. B., *J.A.M.A.*, 1948, v137, 1005.

7. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, 1932, v97, 465.

8. Lewis, J. H., and Davidson, C. S., unpublished observations.

¶ The lot of ACTH used was reported to contain, on different assays, between 0.14 and 1.3 units of pressor activity.

normal adrenal cortical function in these patients with scurvy. The observation, however, is amenable to other interpretations. First, ascorbic acid may not be necessary for those functions of the adrenal cortex studied. This interpretation is supported by the report of Long(9) that the injection of ACTH to scorbutic guinea pigs with but 4% of normal adrenal ascorbic acid remaining resulted in the usual fall in adrenal cholesterol and the development of lymphopenia. Secondly, although scorbutic, the patients studied may have had residual ascorbic acid in their adrenals.

It is impossible to decide between these alternatives at present. It is known, however, that scorbutic guinea pigs retain small but significant quantities of ascorbic acid in their adrenals(10). The same may be true in human scurvy. Moreover, patients dying of scurvy sometimes present a syndrome of hypotension and shock resembling an Addisonian crisis(11). Perhaps only terminally do the adrenals become completely deficient in ascorbic acid.

Summary. 1. Test doses of ACTH were administered to 5 patients with classical scurvy to assess their adrenal cortical activity. The test was repeated in 4 of these patients after therapy with ascorbic acid. 2. The blood eosinophil response to ACTH was that expected of individuals with normal adrenal function and did not change following treatment with ascorbic acid. 3. The urinary excretion of uric acid after ACTH was uninterpretable because of low urine volumes. 4. The blood glutathione, and the serum sodium concentrations were normal or low in these patients, but were unaltered by ascorbic acid therapy. Serum potassium concentrations were normal before and after ascorbic acid therapy. 5. To explain these evidences of normal adrenal cortical activity in scurvy, it may be postulated that either ascorbic acid is not necessary for these adrenal functions, or there was residual ascorbic acid in the adrenals of these patients.

Addenda. Dr. Frank H. Gardner, Peter Bent Brigham Hospital, made a similar study of one patient, a 76-year-old bachelor, with scurvy. A 48-hour ACTH test[||] was done, giving 40 mg of ACTH daily, divided into 4 equal doses. A normal eosinophil response and increase in 17-ketosteroid excretion was observed.

	Control	48 hr
Eosinophil count per cu mm	27	0
17-ketosteroid excretion mg/24 hr	2.3	13.6

The patient presented in addition to clinical evidence of scurvy, the defect in tyrosine metabolism reported in the disease.[**]

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10. Robertson, W. V., Dalton, A. J., and Heston, W. E., *J. Nat. Cancer Inst.*, 1949, v10, 53.
11. Gardner, F. H., personal communication.

[||] Thorn, G. W., and Forsham, P. H. Clinical Tests for Adrenal Cortical Reserve. Progress in Clinical Endocrinology, 1950, Grune & Stratton, New York.

[**] Rogers, W. F., and Gardner, F. H., *J. Lab. and Clin. Med.*, 1949, v34, 1491.

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Failure of Aureomycin and Terramycin to Increase Resistance to Toxic Action of Influenza Virus.[*] (18251)

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Acute toxic damage is induced by the influenza virus in tissues which will not support its multiplication(1). When injected intravenously, intra-abdominally or intra-

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[*] Aided by a grant from the United States Public Health Service.

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cerebrally, the virus may cause death even in animals which are not ordinarily susceptible to influenza infection(1). The toxic action of the influenza virus has caused hepatic, splenic, cerebral and probably myocardial damage(1,2), and pathologic changes have been demonstrated in organs outside of the respiratory tract in fatal cases of influenza A infection(3). It is possible, therefore, that certain of the clinical manifestations of influenza are due to the direct toxic action of the virus. The administration of aureomycin or terramycin to patients with severe cases of proved influenza is usually followed by prompt defervescence and relief from myalgia, fever and other clinical evidences of toxicity(4,5). However, influenza has been a relatively mild disease in recent years and equally rapid terminations of the disease have been observed in the absence of specific therapy(5,6). Aureomycin(7) and terramycin(5,8,9) do not inhibit the growth of the influenza virus in experimental animals although terramycin, under certain conditions, may affect growth of the virus in chick embryos to a slight extent(5,8,9). It has been suggested that these antibiotics may diminish the toxicity of the virus to the host and that this may explain their possible clinical effectiveness in the treatment of influenza. Acute toxicity induced by the intravenous injection of rickettsiae into mice is not prevented or reduced by therapeutic doses of terramycin and aureomycin(10). Likewise, aureomycin and penicillin do not affect the toxicity of viruses of the psittacosis-lymphogranuloma group for mice(11).

In the present study, terramycin and aureomycin were administered to rats and mice, and concentrated suspensions of influenza virus were then injected, to determine if the toxicity of the virus was diminished by the antibiotics.

Materials and methods. The PR8 strain of influenza virus was used throughout, and was propagated in the allantoic fluids of 10- or 11-day-old chick embryos by the usual methods(12). The virus was concentrated 10- to 20-fold in the original allantoic fluid by adsorption on and elution from embryonic erythrocytes. The ID_{50} of the resulting viral concentrates was between $10^{-7.6}$ and $10^{-8.0}$ when titrated in 10-day-old chick embryos. Aureomycin hydrochloride buffered with sodium glycinate‡ and terramycin hydrochloride¶ were used throughout but the dosages are expressed in terms of their respective free bases. Fresh solutions were prepared in saline for subcutaneous injection and in water for oral administration. The subcutaneous dose was 2.5 mg in 0.5 ml given twice daily to mice, and twice this amount, given every 4 hours to rats. The oral dose was 10 mg in 0.2 ml administered twice daily directly into the stomachs of the mice.

White Swiss male mice of the Tumblebrook Farm strain, weighing 18-22 g were used. In a few observations, female mice were found to be as susceptible as males to the toxic action of the influenza virus. The mice were divided into groups of 10 and were given either terramycin, aureomycin, or saline twice daily until the time of their death. Immediately after the second ad-

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‡ Supplied by Dr. Stanton M. Hardy of Lederle Laboratories.

¶ Furnished by Dr. Gladys L. Hobby of Chas. Pfizer & Co.

ministration of antibiotic, 1.5 ml of concentrated virus per 100 g body weight were injected intravenously. In one series, the virus was not injected until after the fourth injection of antibiotic. Control animals were given antibiotics but no virus. The amount of virus used resulted in the death of 50-70% of mice within 24 hours. Only mice succumbing during the first 24 hours after injection of the virus were considered to have died of toxemia.

The rats were males of the Sprague-Dawley strain, weighing 100-150 g. Groups of 5 rats were injected subcutaneously with one of the antibiotics or with saline every 4 hours until death occurred or the experiment was terminated. The virus was injected intravenously after the second dose of antibiotic, using the same dose as in mice. In the rats, this amount of virus regularly produced prostration in 6-8 hours and death in 9-14 hours.

Other groups of control mice and rats were immunized by intravenous injection of sublethal doses of virus 2 weeks before they were challenged.

Results. The immunized mice and rats were completely protected against otherwise lethal doses of the influenza virus. The control animals which received antibiotic but no virus showed no adverse effects save for slight hyperirritability and ruffled fur. There was no significant prolongation of life in rats (Table I) or mice (Table II) treated with aureomycin or terramycin, as compared with animals receiving saline alone. Neither subcutaneous nor oral administration of the antibiotics was effective.

TABLE I. Effect of Aureomycin and Terramycin on Toxicity of Influenza A Virus to Rats.

Substance inj.	Result				
Saline	9,	9,	10,	11,	12*
Aureomycin	8,	9,	10,	10,	14
Terramycin	9,	9,	9,	11,	12
Immune	S,	S,	S,	S,	S

Test animals each received 1.5 ml of virus concentrate per 100 g of body wt intravenously.

Control rats receiving antibiotic and no virus all survived.

* Hours after virus was inj. when death occurred.

S = Survived.

TABLE II. Effect of Aureomycin and Terramycin on Toxicity of Influenza A Virus to Mice.

Substance inj.	Route	No. dead/No. inj.
Saline	Subcut.	8/10
Aureomycin	"	9/10
Terramycin	"	8/10
Saline	Oral	6/10
Aureomycin	"	7/10
" *	"	8/10
Terramycin	"	6/10
" *	"	8/10
Immune		0/10

Test animals each received 1.5 ml of virus concentrate per 100 g of body wt intrav.

Control mice given aureomycin and terramycin alone did not succumb.

* Virus inj. after the 4th dose of antibiotic. In the other groups, virus was inj. after the second dose of antibiotic.

Discussion. The failure of aureomycin and terramycin to alter the toxic action of the influenza virus, the lack of any effect of either of these antibiotics on influenza viral infections in mice(5,7-9), and the failure at least of aureomycin to affect multiplication of the virus in chick embryos(5,7) all would seem to indicate that any clinical benefits derived from the use of these antibiotics in the treatment of influenza are probably not the result of an effect on the virus or on the capacity of the host to resist its toxic action. The clinical aspects, therefore, require further investigation.

It has been suggested that some of the toxic effects of the influenza virus may be due to incomplete and atypical replication of viral particles in tissues in which the virus is not ordinarily considered to have multiplied(13). After intracerebral injection of a non-neurotropic strain of influenza virus, complement-fixing and hemagglutinating substances in the cerebrum of the mouse increased, but infectivity titers did not increase. If this observation should prove to be generally applicable to all tissues in which the titer of infectivity of the influenza virus does not increase, the findings presented here may simply reflect the failure of aureomycin and terramycin to interfere with viral multiplication in the experimental animal. It has not been demonstrated, however, that

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13. Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 541.

atypical replication of the influenza virus will occur in tissues other than the brain of the mouse, or in any of the tissues of the rat, which is not susceptible to influenza infection.

Summary. Aureomycin and terramycin, administered subcutaneously and orally, did not increase the resistance of mice or rats to the acute toxic action of influenza A virus.

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Mustard Inactivated Rabies Vaccine. (18252)

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Our preliminary paper(1) on the production of vaccines by treating virus suspensions with mustard was based in part on studies in this laboratory on the effect of mustard on proteins. These studies(2), which were published subsequently to our paper, showed among other things that relatively small amounts of mustard inactivated enzymes and that mustard reacted with the carboxyl groups of protein molecules but not with the free amino groups. After our preliminary paper, one of us(3) studied the action of mustard on animal, plant, and bacterial viruses, as well as on bacteria, yeasts, and a pneumococcus-transforming agent, and found that viruses and cells were inactivated faster than enzymes and that viruses containing desoxyribose nucleic acid were inactivated faster than those containing ribosenucleic acid. Mustard gas seems an ideal inactivating agent because its action is complete within a relatively short time and in our experiments the end products were not toxic. Mustard is bactericidal; and if the contamination of the starting material is not too great, sterile vaccines are secured. It is in fact much more like ultraviolet light in its action than like most other chemical agents. When tested for antigenicity it is found that inactivated rabies and equine encephalomyelitis viruses immunize when diluted 10^{-3} or higher, hog cholera and Newcastle viruses immunize when undiluted, while very preliminary experiments with pseudorabies and Baker's cat viruses have failed to show antigenicity.

In the present paper some experiments made with inactivated rabies virus are reported, since the vaccine promises to be of practical importance and since the findings may be useful as a guide for the preparation of other vaccines.

Materials and methods. The fixed viruses used were a strain obtained from the Bureau of Laboratories of the Department of Health of the City of New York, through the kindness of Dr. Jules Freund, the Pitman-Moore strain 980 from Dr. R. B. Gochenour, and a strain adapted to mice from Dr. Harald Johnson. The street virus used for challenging the guinea pigs also came from Dr. Johnson. Rabbits, guinea pigs, and mice were inoculated intracerebrally and the brains removed when the animals were completely paralyzed. Small amounts of brain material were suspended in water, using a glass grinder; larger amounts were suspended in water in a Waring blender. Water was used rather than saline as the reaction is retarded by halid ions.

Test of immunity. The least amount of antigen injected into the peritoneal cavity that would immunize guinea pigs weighing 250-300 g against street virus was determined. This test has been described in some detail(1).

Mustards. Sulfur mustard (*bis* (β-chloroethyl) sulfide) was prepared in this laboratory by the method of Bent(4) followed by fractional crystallization. The final product melted at 14.4°C. The 3 nitrogen mustards were obtained from Edgewood Arsenal.

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1. TenBroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 271.

2. Herriott, R. M., Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1946, v30, 185.

3. Herriott, R. M., *J. Gen. Physiol.*, 1948, v32, 321.

Inactivation with sulfur mustard. A saturated solution was obtained by adding 0.01 ml of mustard to each 15 ml of sterile distilled water taken immediately from the refrigerator at an approximate temperature of 3°–10°C. This gives a concentration of about 5×10^{-3} molar. The distilled water is contained in a glass-stoppered cylinder, and in introducing the mustard the pipette is kept from the sides of the cylinder and the tip is placed below the surface of the water so that the mustard sinks. The cylinder is immediately closed and shaken vigorously for approximately 30 seconds. An equal part of the saturated solution is added to a 20% brain suspension warmed to 25°C, giving a 10% brain suspension half saturated with mustard. If lower concentrations are needed, an appropriate amount of distilled water is added to the brain suspension, then the proper amount of saturated mustard, so that the final concentration of brain is always 10%. The reaction of the brain suspension is adjusted to pH 7.3 before mixing with the mustard solution. In its reaction with water mustard produces acid and the pH of the mixture is determined for the first ½ hour by the drop method, and normal sodium bicarbonate sterilized by filtration is added dropwise to keep the pH at approximately 7.3. After 2 hours at 25°C the preparations are placed in the refrigerator.

Precautions to prevent burns with mustard. The mustard is kept in a glass-stoppered cylinder. Immediately after use the contaminated pipettes are placed in alcohol and then washed in warm water for 15 minutes before they are transferred to cleaning fluid. The cylinders used are treated in the same way. In the more than 3 years work there has not been a single burn.

Tests for inactivity of the virus. The routine test for inactivation has been the intracerebral injection of 0.1 ml of the 10% treated material into each of 3 guinea pigs the day after the preparation of the vaccine. These animals are observed over a period of a month. Further tests for the presence of active virus will be described below.

TABLE I. Protection by Inactivated Vaccines.

Source of brain suspension	No. tested	Protection of 50% of the guinea pigs by 1 ml of a concentration of		
		.008% (excellent)	.04% (good)	.2% (poor)
Guinea pig	6	1	2	3
Mouse	7	5	1	1
Rabbit	20	3	11	6

Results. With rabies and equine encephalomyelitis viruses it has been repeatedly shown that the best vaccines are those produced by the least amount of mustard that will inactivate. When the starting material is fixed virus in rabbit brain, ½ saturation always inactivates a 10% suspension, whereas 1/4 saturation will rarely do so. The ½ saturated material is a much better antigen than that produced by complete saturation. When 5% mouse brain is used a 1/4 saturated preparation is inactive and is a better antigen than that produced by ½ saturation. Thirty-three preparations have been treated with mustard and their antigenicity determined. The results are given in Table I and show that while all of them immunize, some preparations are better than others. Guinea pig brain although it has a high virus content does not make a good antigen when tested by the method used. Mouse brain is almost invariably good, and rabbit brain varies considerably. The best rabbit brain preparations we have had were from suspensions made in other laboratories where large numbers of rabbits were inoculated and the brains combined. In our laboratory, where small numbers of rabbit brains were combined and treated with mustard, most of the resulting antigens could be classed as good.

Comparison of mustard inactivated vaccines with those inactivated with phenol or irradiated light, and with untreated fixed virus. In order to compare the mustard preparations with those inactivated by other means we secured the help of 2 outside laboratories that were producing vaccines by the

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4. Bent, H. E., *Science,* 1947, v106, 374.

TABLE II. Comparison of Antigenicity of Rabbit Brain Fixed Virus Inactivated by Phenol and Mustard.

Date of preparation	50% end point in mice before inactivation $.03 \times 10^{-}$	Age of vaccine when tested, days	Inactivated by	% suspension inj. intraperitoneally (1 ml) and results of challenge with street virus intramusc. 3 wk later*			Controls
				0.5	0.1	0.02	
5/8/47	7.1	99	Phenol	7/7†	5/5	5/5	7/7
			Mustard	0/6	1/5	1/5	
				0.5	0.2	0.05	
6/17	6.8	22	Phenol	6/6	4/6	6/6	6/6
			Mustard	0/6	3/6	5/6	
				0.2	0.04	0.008	
9/23	6.67	52	Phenol	0/5	3/5	5/5	5/5
			Mustard	1/5	2/5	3/5	
				0.2	0.04		
6/8/48	7.5	112	Phenol	2/5	5/6		5/6
			Mustard	1/6	2/6		
				0.2	0.04		
9/28	7.8	30	Phenol	4/6	3/6		5/6
			Mustard	0/6	5/6		

* In Tables III, V, and VI, this heading has been shortened to "Results of challenge."

† In all tables, numerator indicates the no. of animals that died; the denominator, no. of animals inoculated.

TABLE III. Comparison of Mustard Inactivated and Irradiated Rabbit Brain Fixed Virus.

Date of preparation	50% end point in mice before inactivation $.03 \times 10^{-}$	Age of vaccine when tested, days	Inactivated by	Results of challenge* .2	.04	Controls
3/5/48	6.5	10	Irradiated	1/5	4/5	4/5
			Mustard	1/5	4/5	
5/3	6.0	10	Irradiated	2/6	2/6	5/6
			Mustard	1/6	5/6	
5/21	6.0	11	Irradiated	2/6	2/6	6/6
			Mustard	0/6	3/6	
1/30/50	6.0	10	Irradiated	2/8	6/8	8/8
			Mustard	2/8	3/8	
4/3	6.17	51	Irradiated	3/8	6/8	7/8
			Mustard:			
			¼ saturated	2/8	7/8	
			½ "	1/8	6/8	

* See Table II.

recognized methods. They provided brain suspension for inactivation by mustard in our laboratory and permitted us to compare it with their own product made on the same day.

Comparison with phenol vaccine. With the assistance of Dr. Jules Freund, 5 different rabbit brain suspensions were secured from the Bureau of Laboratories of the Department of Health of the City of New York, treated with mustard, and then compared with the phenol inactivated vaccines made in the New York City laboratories on the same day. The results are given in Table II and show that the mustard inactivated preparations are invariably better than those inactivated by phenol.

Comparison with irradiated vaccine. Similar comparisons were made between irradiated and mustard inactivated preparations with the help of Drs. Hilleman and Nigg of E. R. Squibb & Sons. The results of the 5 tests, given in Table III, show that irradiated and mustard treated antigens have about the same antigenicity when tested on guinea pigs. It should be added that in the

TABLE IV. Comparison of Active Fixed Virus and the Same Virus Inactivated with Mustard as Immunizing Agents.

Virus strain	Brain suspension of	Vaccination with virus	Intracerebral titer in mice after freezing .03 × 10–	% brain in virus or vaccine and results of challenge with street virus intramusc. 3 wk after vaccination				Controls
				.1	.01	.001	.0001	
Georgia	Mouse	Active	5.76	—	0/5*	4/8	8/8	6/8
		Mustard inactivated		0/7	1/8	—	—	
Gochenour	Rabbit	Active	5.4	—	6/8	8/8		6/8
		Irradiated		3/8	—	—		
		Mustard inactivated		5/8	—	—		
				.2	.04	.01		
Gochenour	Rabbit	Active	5.6	—	4/7†	6/8		7/8
		Mustard inactivated:						
		¼ saturated		2/8	7/8			
		½ saturated		1/8	6/8			

* 3 of 8 guinea pigs died of fixed virus before the challenge.
† 1 of 8 guinea pigs died of fixed virus.

Squibb laboratories, Habel potency tests were done on these vaccines and all of the irradiated material passed the test while only one of the 5 mustard vaccines passed.

Comparison with untreated fixed virus. In 3 experiments, the results of which are given in Table IV, the immunizing action of active fixed virus is compared with that of the same virus inactivated with mustard. The active virus was placed in ampules and frozen with dry ice in alcohol, then kept under dry ice until the test injections were made. The results show that fixed virus has about 5 to 10 times the immunizing action of mustard inactivated material. It should, however, be noted that the 0.01% of fixed virus which immunized solidly was near the minimal infectious dose as some of the guinea pigs died from fixed virus infection before the challenge.

Other types of mustards. Preliminary tests have shown that the nitrogen and methyl sulfur mustards will inactivate fixed virus without destroying all antigenicity. Careful comparative tests between the vaccines produced by these and ordinary mustard have not been made. For the preparation of large amounts of vaccine the nitrogen mustards have the advantage that they can be weighed and that they are readily soluble in water. Sulfur mustard has to be shaken vigorously to saturate water and this is difficult with amounts of over 1 liter. However, it can be dissolved in alcohol or one of the "cellosolves" and then added to the water.

Toxicity of the vaccine. When a vaccine is prepared with a substance such as mustard, the first question that arises is whether the resulting preparation is toxic. It is well known that sulfur mustard disintegrates in water to yield a small quantity of a toxic material(5). However, no evidence of toxicity or irritation has been noted following intracerebral or intracutaneous injection of 0.1 ml undiluted vaccine into guinea pigs, or after intraperitoneal or subcutaneous injection of 1 ml of the same; after intraperitoneal or subcutaneous injection of 5 ml undiluted vaccine into dogs; or after intraperitoneal or intracerebral injection of 0.1 ml vaccine diluted to 1% brain suspension into mice. No tests have been made on man.

Drying of the vaccine. When frozen and dried, there is considerable loss of antigenicity, as shown in Table V. However, once dried, the antigenicity, as would be expected, remains unchanged for a long period. In 2 such samples tested there was no change over a period of 2 years in samples kept in the refrigerator.

Stability of the vaccine. As shown in Table VI, the vaccine is quite stable at refrigerator temperatures. Two of the samples tested more than a year after preparation

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5. Stein, W. H., Moore, S., and Bergmann, M., *J. Org. Chem.*, 1946, v11, 664.

TABLE V. Effect of Freezing and Drying on Vaccines.

Vaccine	Results of challenge*			Controls
	.2	.04	.008	
	A.			
Original	0/5	3/5	4/5	
Frozen and dried	3/5	5/5	4/5	
	B.			5/5
Original	0/5	0/5	2/5	
Frozen and dried	0/5	3/5	3/5	

* See Table II.

TABLE VI. Keeping Properties.

Vaccine	Interval between preparation and test	Results of challenge*		Controls
		.1	.04	
Guinea pig brain	110 days	1/5	1/5	7/7
	250 ″	0/5	4/5	5/5
		.2	.04	
Rabbit	99 days	1/5	1/5	7/7
	245 ″	0/5	4/5	5/5
″	15 days	0/6	0/6	6/6
	1 yr. 7 mo.	0/6	0/6	4/5
″	11 days	0/6	4/6	6/6
	91 ″	0/6	2/6	6/6
	1 yr. 3 mo.	0/6	1/6	4/5

* See Table II.

were almost as active as at the time of their first test. The other 2 samples showed some loss of antigenicity but they were still good immunizing agents.

Tests for active virus. As noted above, some of the vaccine preparations are extremely active, immunizing when diluted 1:1000 or more, and the question arises: Is active virus present which can be demonstrated by tests other than the usual one of intracerebral inoculation of undiluted vaccine? It has been impossible to show that active virus is present by inoculating guinea pigs intracerebrally with a good vaccine, removing the brains from these animals at 2, 4, and 6 days, and testing for virus by intracerebral injection into normal guinea pigs. Furthermore, blind passage from such animals in guinea pigs has failed to demonstrate virus. No evidence for a mutant that was avirulent but antigenic was obtained. No active virus could be detected from a vaccine that had a high immunizing property and that was diluted in distilled water 1:10, 1:100, and 1:1000, and after standing for 1 hour in the refrigerator was injected intracerebrally into mice. Andrewes and Elford (6) reported that when ectromelia irradiated vaccine is mixed with active virus and the mixture injected intraperitoneally, no infection results. In other words, the active virus is masked or interfered with. They suggest that the activity of some vaccines may be due to masked virus. Their experiment was repeated, using rabbit brain virus inactivated with mustard, as well as the same brain inactivated by ultraviolet light. The vaccines were diluted so that they contained 5% brain suspension, and to 0.9 ml of this vaccine was added 0.1 ml of known dilutions of fixed virus in milk. Mice were inoculated intracerebrally with 0.03 ml of these mixtures. Ten mice were inoculated with each, but the brain suspension itself was quite toxic and many of the mice died immediately. The results of the experiment are given in Table VII and it is clear that there was no masking effect by either the mustard or light inactivated vaccine.

Neutralizing antibodies. The activity of vaccines has been judged by their ability to protect guinea pigs against street virus, but some tests have been made for the presence of neutralizing antibodies. When low dilutions of these vaccines are injected, neutralizing antibodies have been detected some 3 weeks later. They have not been detected when higher dilutions, such as 1:250 or 1:1000, have been used, yet such dilutions have protected the guinea pigs. The test for immunity is more sensitive than the test for neutralizing antibodies 3 weeks after vaccination.

Discussion. There are a great variety of mustards and some or all of them may be used to produce vaccines. It is possible that one or more of these are better than sulfur mustard, but more is known about the last and it is the one to be preferred at present. It seems to us remarkable that every virus so far tested has been inactivated with mustard and with those examined in detail there has been retention of some immunizing

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6. Andrewes, C. H., and Elford, W. J., *Brit. J. Exp. Path.*, 1947, v28, 278.

TABLE VII. Summary of Experiment to Determine whether Vaccine Would Act as an Interfering Agent when Mixed with Active Virus.

Vaccine Rabbit brain	Dilution of virus in 5% vaccine					Vaccine controls
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Inactivated with mustard	6/6	8/8	8/9	2/8		0/7
Irradiated, Squibb	9/9	10/10	8/9	5/6		0/8
Control. Virus diluted in milk		10/10	5/10	1/10	0/10	

property. Some of these antigens have been excellent while others will not immunize if diluted. However, the method should be useful in producing antigens to some of the viruses that produce disease in man. It also seems noteworthy that highly active vaccines have been produced against rabies from starting material of not especially high titer. It may be that the titer does not necessarily measure the amount of virus protein. If active brain material is suspended in strong buffer at pH 8 and titered immediately and again after 1 or 2 hours, it is found that the second titer is appreciably higher than the first, suggesting that the virus is aggregated and that in the buffer there is some dispersion. It is also possible that part of the antigenic material is not active virus but a precursor.

Although a relatively small amount of mustard is used to inactivate viruses and from isotopic studies it was found that a large proportion of the mustard reacts with the water, a rough calculation indicates that there must be several thousand mustard particles reacting with each virus particle. However, this is a reflection of the large particle size of the virus for on a percentage basis the amount bound is small. Certain reactions of mustard with simple proteins are understood but no comparable information is available for the reactions with nucleic acids or nucleoproteins.

Summary. The above work shows that mustard gas added to brain suspensions from animals infected with fixed virus gives a vaccine which by the method of testing used is better than that produced when the same brains are inactivated with phenol, about equal to that when the inactivation is by ultraviolet light, and almost as good as active fixed virus. The vaccine is very stable at refrigerator temperatures, and the method of preparation is simple and requires no unusual laboratory equipment.

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Prevention of Street Virus Infection by Vaccine.* (18253)

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Webster(1), in a review of the literature up to 1936, concluded that there is no good experimental evidence that rabies vaccine given after the injection of street virus will prevent the development of the disease. Since that date we have not found published experiments on this phase of rabies immunization. In the preceding paper(2) the evaluation of rabies vaccines was made by challenging guinea pigs 3 weeks after the intraperitoneal injection of known dilutions of

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* The painstaking and accurate technical assistance of Mrs. Laura Lee Crosby and Mr. George Quabeck greatly aided the rabies work in this laboratory.

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1. Webster, L. T., *Am. J. Hyg.*, 1939, v30, 113.

2. TenBroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 523.

TABLE I. Relation of Amount of Vaccine to Development of Immunity.

Interval between vaccination and challenge, wk	Dilution of vaccine inj. and results				
	1:10	1:50	1:250	1:1250	1:6250
3	—	0/8*	0/8	4/8	6/8
2	—	1/8	0/8	2/8	—
1	2/8	1/8	4/8	—	—
Controls—8/8					

* In all tables, numerator is the No. of animals that died; denominator the No. inoculated.

TABLE II. Immunity in Relation to Time Between Injection of Vaccine and Street Virus.

No. of inj.*	Dilution of vaccine	Time of inj.	Results
1	1:5	24 hr before virus	0/10
1	1:5	2 hr after virus	4/10
1	1:5	24 hr after virus	4/10
5	1:25	2 hr, 1, 2, 3, and 4 days after virus	3/10
Controls			9/10

* 1 ml of vaccine inj. intraper.

vaccine. Experiments were made with shorter intervals between the challenge and the vaccine injection. In the experiment reported in Table I the results are somewhat irregular, but they confirm earlier experiments in showing that the shorter interval between the vaccination and the challenge, the larger the amount of vaccine necessary to protect.

Experiments were made to see whether vaccine injected after the street virus would protect guinea pigs. The methods used have been described(3). The vaccines were fixed virus inactivated by mustard(2). The challenge virus was a strain of street virus secured locally‡ and passed twice through guinea pigs by intracerebral inoculation, the second passage being used. This virus was selected rather than the one used in the test of immunizing ability because it had a somewhat longer incubation period. Guinea pigs weighing between 300 and 400 g were used. Twenty guinea pigs were inoculated intramuscularly with 1 X 10^{-3} dilution in milk of a 10% brain suspension of the second passage street virus. Three hours later half of them were given an intraperitoneal injection of 1 ml of a 1:5 dilution of 10% rabbit brain vaccine ½ saturated with mustard 3 months previously. This injection was repeated at 24 and 48 hours. Eight of the 10 control animals developed rabies while all 10 of those given vaccine were protected. This experiment has been repeated with variations and with different vaccines; and while not all of the vaccines have protected guinea pigs, there is no question but that experimental evidence has been provided that the development of rabies can be prevented. The intervals between the vaccine and the virus were varied, as shown in Table II. Five per cent mouse brain 2/10 saturated with mustard 1 month previous to the experiment was used.

The experiment shows that vaccine given before the virus is injected gives better protection, that it makes little difference whether the vaccine is given shortly after the virus or a day later, and that one injection of vaccine gives the same or better immunity than an equal quantity given over a period of days. This type of experiment has also been repeated and these three results have been confirmed.

Route of injection of vaccine. In this experiment a mouse brain vaccine prepared 6 months previously and a rabbit brain vaccine prepared 5 months before the experiment were used, and the protecting action following intraperitoneal, subcutaneous, and intramuscular injection was compared. The virus was given 1 hour after the vaccine, and in the intramuscular injections the virus was given into the left leg and the vaccine into the right.

It will be seen in Table III that the mouse

TABLE III. Effect of Route of Injection of Vaccine on Protection Against Street Virus.

Vaccine*	Route	Results
Mouse brain	Intraperitoneal	3/8
	Intramuscular	4/8
	Subcutaneous	3/8
Rabbit brain	Intraperitoneal	3/8
	Intramuscular	6/8
	Subcutaneous	8/8
Controls		6/8

* 1 ml of a 1:5 dilution injected 1 hr before the virus.

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3. TenBroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, v62, 271.

‡ From Dr. R. S. Edmonds, Princeton, N. J.

TABLE IV. Sensitization to Street Virus by Vaccine.

Vaccine				No. showing symptoms by day										
Brain of	Mustard	Route of inj.	Dilution	8	9	10	11	12	13	14	15	16	17	Deaths
Rabbit	4/10 saturated	Subcut.	1:5*	0	0	1	3	7	7	7	7	7	8	8/8
”	1/4 ”	”	1:5*	0	0	0	3	4	6	7				7/8
Controls				0	0	1	1	2	3	3	4	6		6/8
Mouse	1/4 ”	”	1:25†	0	2	4	6	7						7/8
”	1/4 ”	”	1:2.5†	1	3	5	6	8						8/8
”	1/4 ”	Intraper.	1:2.5†	0	4	5	6	7						7/8
Controls				0	1	3	3	3	5	5	5	5	6	6/8

* 1 ml of vaccine inj. 1 hr before virus.
† 1 ml of vaccine inj. 24 hr after virus.

brain vaccine protected to the same extent by all 3 routes, whereas the rabbit brain injected subcutaneously or intramuscularly had no protecting action. The intraperitoneal injection of rabbit brain vaccine did however have some protecting action. That vaccines differ, some having a much better protecting action when injected intraperitoneally than subcutaneously, has been repeatedly confirmed. Other vaccines give as good or better protection subcutaneously than they do after intraperitoneal inoculation. Most of these results have been obtained in tests made when the vaccine was injected 3 weeks previous to the virus injection. This difference in immunizing ability is apparently due to the physical state of the vaccine, for it is not connected with the animal brain used. Some mouse brains give better protection by intraperitoneal injection and others by subcutaneous, and the same variation is found with different rabbit brain preparations. It is a matter that requires further study before an explanation can be given.

Sensitizing action of the vaccine. In the above experiment there were more deaths in the guinea pigs given the subcutaneous injection of rabbit vaccine than in the controls, a result that has repeatedly occurred, suggesting that the vaccine had made them more sensitive to the virus. In Table IV is shown the time of appearance of symptoms in the guinea pigs injected subcutaneously with rabbit brain, taken from the experiment given in Table III with the addition of one set of animals used in the same experiment but not included in the above table and an experiment with a moderately active mouse brain preparation. It will be seen that symptoms appeared earlier and that there were more deaths in the vaccinated groups than in the controls. This is an effect that was sometimes observed in animals that were given vaccine 3 weeks before the challenge.

TABLE V. Sensitization to Street by Fixed Virus.

	No. showing symptoms of street virus by day						
Vaccine	8	9	10	11	12	13	Deaths
Fixed virus, intraper. inj., 1 ml, 10^{-4} 10% suspension 3 wk before inj. of street virus	4	8					8/8
Controls	2	3	3	5	5	6	6/8

Fixed virus not inactivated injected 3 weeks before the challenge brings out this sensitizing effect in a more striking manner, as shown in Table V. In this experiment, on the 9th day after challenge all 8 of the vaccinated animals showed symptoms, whereas in the control animals only 6 of 8 developed rabies and the longest incubation period was 13 days.

Discussion. All of these experiments have been made on guinea pigs and there may be some question as to how far the results can be applied to man, but some of the findings at least confirm the thinking of those who are concerned with human vaccination. Experimental evidence is presented that rabies can be prevented by vaccine given after the injection of the virus, provided a vaccine of high immunizing ability is used and in sufficient quantity. This is brought out in spite of the fact that the incubation period in the guinea pig is approximately 10 days, a much shorter time than in the larger animals. If man were to develop immunity as rapidly as the guinea pig—and it is believed that this prevention is immunity and not interference—the results would encourage fewer injections of vaccine in larger amounts than is the current practice.

The findings suggest that a part, at least, of the antigen is incorporated in the immune body, an idea that is not new but one that has not been confirmed. It is desirable that more work be done on this subject.

For evaluating vaccine to be used in man the power to protect after the injection of virus has advantages that merit consideration. It is this property of a vaccine that we want to measure, and furthermore the results are known in a short time. Extensive comparative tests have not been made but it appears that the vaccines that have a high protective value when given 3 weeks before the challenge are the ones that give protection when injected at the same time as the virus.

That guinea pigs may be sensitized to virus by vaccines, with a resulting shorter incubation period and more infections than in the controls, is a disturbing fact. In the past since the use of inactivated vaccines was begun, great numbers of individuals have been injected with products that had little immunizing ability. It is not likely that it can be determined whether man was in some instances made sensitive to the virus, but the findings emphasize the need, which is generally realized, for better vaccines and should stimulate efforts to concentrate the immunizing substances in vaccines, with the elimination of unnecessary proteins. Many experiments have been made in our laboratory and elsewhere on this problem. We have succeeded in eliminating some of the extraneous proteins without loss of immunizing ability but have not succeeded in concentrating the immunizing substance. All such experiments have been made with active virus that was later treated with mustard. In the preceding paper(2) it is shown that mustard inactivated virus retains its immunizing ability over a long period of time when stored in the refrigerator, and it now seems that this is the material to attempt to purify rather than the active virus which is so unstable.

Summary. Guinea pigs inoculated intramuscularly with street virus can be protected by mustard inactivated fixed virus injected at the same time or 24 hours later, provided the virus has a high immunizing titer. These results give experimental proof that vaccine will protect after infection and also provide a test that could be used in evaluating vaccines for human use. If non-protecting amounts of vaccine or fixed virus are injected, the incubation period may be shorter and the death rate slightly higher than in the control animals receiving only street virus.

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Influence of APF and Aureomycin on the Growth of Dairy Calves.* (18254)

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Wallace and Loosli(1) have reported on a series of diets which can be used to replace successfully fluid whole milk in feeding dairy calves after they are 10 to 14 days of age. In these studies it was observed that generally the rates of growth on the milk substitutes were not as rapid during the first month as on whole milk and that the calves

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* Aided by a Grant from Coop. G.L.F. Exchange, Ithaca, New York.

1. Wallace, H. D., Loosli, J. K., and Turk, K. L., *J. Dairy Sci.*, in press.

TABLE I. Effect of APF on Growth Rate and Incidence of Diarrhea in Dairy Calves to 8 Wks of Age.

Trial	Diet	No. of calves	Avg daily gain on APF, lb.	Avg daily gain on No APF, lb.	Avg incidence of scours APF, days	Avg incidence of scours No APF, days
I	10	8	0.88	0.81	1.0	4.5
	11	8	1.09	1.01	0.0	2.7
	12	8	1.08	0.94	1.0	1.0
	Milk	8	1.20	0.95	0.2	2.7
II	11	6	1.15	0.67	12.7	21.3
	13	8	1.07	0.94	5.5	12.2
	Milk	8	1.22	1.02	0.2	7.5
		54	1.11 ± .04	0.92 ± .04	2.5	6.9

consistently showed more diarrhea. Following the report by Cunha *et al.*(2) that APF supplements increased the growth rate of pigs fed a diet containing adequate amounts of vitamin B_{12}, a series of experiments was conducted to test the effect of an APF supplement upon the growth rate and incidence of scours in dairy calves.

Experimental. The diets used and the methods of feeding and management have been reported elsewhere(1). The milk substitutes all contained 50 to 70% of dried skim milk or dried whey or a combination of the two. Mixture 11 was composed almost entirely of dried whey. The mixtures were suspended in water and fed as a drink to replace the fluid whole milk during the second to eighth weeks. Hay and a grain mixture were allowed *ad lib* and the amounts consumed were recorded. In trial I the APF supplement† was added to the milk substitute at the rate of 56 lb per ton. This provided approximately 10 g of APF per kilogram of total dry matter intake. It was also stirred into the whole milk at each feeding to give the control group the same amount as calves fed the substitutes. In trial II crystalline aureomycin† was added to the milk substitutes at the rate of 10.0 g per ton and added to the whole milk to furnish an equal daily intake. In all comparisons the calves were selected by pairs at 1 to 4 days of age according to size, age and sex and placed at random on the APF supplement or held as negative control.

Results and discussion. The results of 2 experiments with a total of 27 pairs of calves are summarized in Table I. Considering all comparisons the calves gained an average of 1.11 lb per day with APF and only 0.92 lb without APF. The difference between these rates of gain is statistically significant ($P<0.01$). There was also a striking difference in the incidence and severity of diarrhea. As an average the calves fed APF showed some degree of undesirable looseness of the feces on 2.5 days during the 8-week study in comparison with 6.9 days for their control pair mates. From estimates of the degree of scouring it became clear that both the APF supplement and the aureomycin reduced the severity as well as the duration of diarrhea, suggesting that an antibiotic effect of aureomycin in the intestinal tract may be primarily involved. One control calf on mixture 11, trial II, died from scours, thus data are shown for only 3 pairs on this diet. It is especially interesting that with every dietary treatment APF feeding showed an advantage.

These results do not agree with the reports of Rusoff and Haq(3) or Williams and Knodt (4). Possibly the differences can be explained on the small numbers of calves used by these workers. The increased rate of gain from

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2. Cunha, T. J., Burnside, J. E., Buschman, D. M., Classcock, R. S., Pearson, A. M., and Shealy, A. L., *Arch. Biochem.*, 1949, v23, 324.

† The APF supplement and crystalline aureomycin were furnished by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y.

3. Rusoff, L. L., and Haq, M. O., *J. Dairy Sci.*, 1950, v33, 379. (Abs.).

4. Williams, J. B., and Knodt, C. B., *J. Dairy Sci.*, 1950, v33, 380. (Abs.)

APF of 20% is not a large increase and, considering variations, would not be significant from a few pairs of calves. The results become impressive with larger numbers, however. Rusoff and Haq(3) used Merck's APF (a vitamin B_{12} supplement) and added it only to the grain mixture, which is usually consumed in rather small amounts by calves during the first 4 to 6 weeks. It is not surprising that no response was observed from vitamin B_{12} under these conditions. The effect observed in our experiments appears to be largely antibiotic since crystalline aureomycin gave a response equal to the APF supplement alone, both on the milk and milk substitute diets. The response observed in these studies appears to agree with the results reported by Dyer *et al.*(5) using pigs.

Summary. Adding an APF supplement (Lederle) and crystalline aureomycin to the diet of young dairy calves significantly increased the rates of growth and reduced the incidence and severity of diarrhea. The data suggest that the responses observed may be largely the result of an antibiotic effect of aureomycin.

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Particulate Component of Plasma from Fowls with Avian Erythro-Myeloblastic Leucosis.* (18255)

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Recently, studies were undertaken in this laboratory directed toward the purification and the determination of the chemical and physical properties of the virus of avian erythro-myeloblastic leucosis. With the progress in the work there has been encountered a particulate component in the blood plasma of birds affected with this disease. The component cannot be identified unequivocally with the virus without quantitative biological correlation based on infectivity measurement. Though investigations are underway for the development of suitable procedures for such correlation, the results will not be available for some time. Meanwhile, the possible significance of the component would appear to warrant a preliminary account of the results thus far observed.

Experimental. The condition studied was the erythro-myeloblastic form of avian leucosis identified as the BAI Strain A(1) obtained from a diseased bird provided by Dr. E. P. Johnson.‡ The disease, characterized by the appearance of exceedingly large numbers of primitive cells in the blood stream, has been maintained for the past year by continual passage either by whole blood or filtered plasma injected intravenously in 0.1 or 0.05 ml volumes in 3-day-old New Hampshire Red chicks. The procedures employed in the isolation and partial purification of the component may be illustrated by those of a typical experiment. Blood was drawn into heparin (10 ml heparin, Liquaemin, "Roche-Organon," 1 ml = 10 mg, per 100 ml blood) from 15 chicks by heart puncture 39 days after intravenous injection of 0.05 ml of filtered plasma each. The individual samples yielded a pool of 255 ml whole blood

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† Predoctorate Fellow of the U. S. Public Health Service, Natl. Cancer Inst.

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‡ The authors wish to express their deepest appreciation for the interest and encouragement of Dr. Johnson in the undertaking of this work.

with a count of $10^{8.9}$ primitive cells per ml and a volume of 124 ml of plasma after removal of cells by low-speed centrifugation. The plasma was passed immediately through 02 Selas (Selas Corporation of America, Philadelphia, Pa.) filter candles under 4 pounds pressure, yielding a volume of 106 ml. The plasma was distributed in 15 ml quantities in celluloid tubes and spun in a chilled head at 20,000 X G in the vacumn type air-driven ultracentrifuge for 60 minutes. The resulting supernate was poured off, and the pellets were resuspended by gentle pipetting in 5.5 ml Simms'(2) solution, pH 7.3. After centrifugation of the suspension in the horizontal head of an International Centrifuge No. 2 at 2300 X G for 15 minutes, the supernate was taken off with a pipette, diluted to 15 ml and spun again in the ultracentrifuge, this time at 15,000 X G for 30 minutes. The supernate was poured off, and the pellets were taken up in 1.0 ml of 0.07 M NH_4Cl-NH_4OH buffer solution, pH 7.3. The suspension was spun in the horizontal head as before, the supernate was taken off and, after dilution to 15 ml with the same diluent, ultacentrifuged for the third time. The sequence of high and low-speed centrifugation was carried through the third and fourth cycle as in the second cycle. The final pellet was taken up in 0.5 ml of the NH_4Cl-NH_4OH buffer solution. In control experiments, identical procedures were applied to plasma from birds failing to develop the disease on injection with the same infectious material and to plasma from birds obtained from a different source receiving no injection. Nitrogen was determined by direct nesslerization(3) and desoxyribonucleic acid (DNA) by the method of Stumpf(4).

Results. The plasma of birds with this disease differs visibly from normal chick plasma, appearing to be of much greater viscosity and seeming to contain relatively large amounts of mucin-like material. Filtration was accomplished without great difficulty, however, 50 ml of plasma passing through each 2-inch candle in about 45 minutes.

The initial high-speed centrifugation yielded large, well-packed pellets, about 8 mm in diameter, with red and brown pigment in the center surrounded by clear, gel-like material at the periphery. With pipetting, most of the material was easily resuspended to give much opalescence, a small amount remaining as visible flakes. Nearly all of the pigment and much colorless material were removed by the low-speed centrifugation. Subsequent cycles resulted in progressive loss of material and increasing transparence of the pellets. The final suspension, approximately 175 X concentrated with respect to the starting volume (taking account of loss due to sampling), was highly opalescent, and no pigmented material was evident.

An electron micrograph of the material of the fourth cycle placed on the screen in the conventional way, washed with distilled water and shadowed with chromium, is shown in Fig. 1. There are seen the images of large numbers of particles of unusual morphology. The majority of particles vary in size and are spheroidal in shape with smooth or slightly roughened surfaces. Many forms are seen, however, which exhibit a definite tail structure varying somewhat both in length and thickness. The form of these particles seems to lie between that of the virus of Newcastle disease(5) and the forms of some of the tailed bacteriophages(6). There are seen also numerous short, thread-like particles which have the appearance of the tail structure possibly torn from the head pieces. The particles without tails exhibit the size and appearance of the heads of the tailed forms. An approximate size range of these heads and particles was 60 to 100 mμ, and the lengths of the tails were from 100 to 200 mμ. While the preparation was by no means homogeneous with respect to the char-

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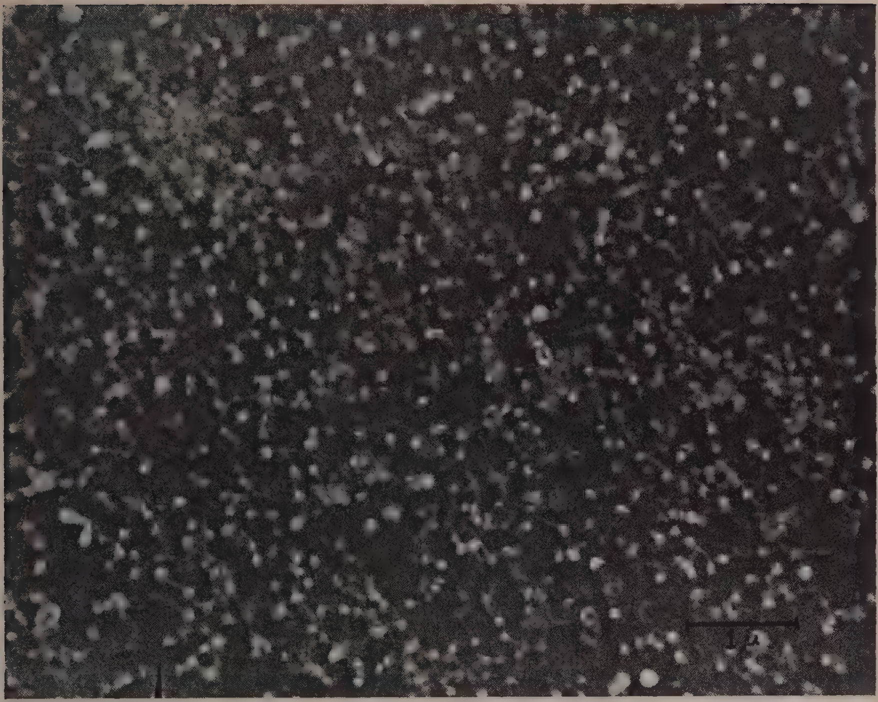

FIG. 1.
Electronmicrograph of a concentrate from the plasma of fowls with avian erythro-myeloblastic leucosis. Magnification 15,000×.

acteristic particles, the latter constituted the major part of the material.

The material sedimented in the analytical ultracentrifuge with a single, though greatly diffuse boundary, at a rate of 630 S, and there was evidence of unsedimentable material in the diagram. Although the preparation contained sufficient material to be opalescent, the absorption of ultraviolet light in the ultracentrifuge seemed relatively low. The yield of nitrogen in the pellet of the fourth cycle taken up in Simms' solution in a similar experiment was 210 μg from 120 ml of plasma. Desoxyribonucleic acid was too small in amount for measurement, but the amount was less than 125 μg. The ratio N/DNA was not less than 1.

No material of this sort was seen in the plasma of birds refractory to infection or in that obtained from birds of comparable age from a source outside the laboratory. Small, clear pellets were observed with the first centrifugal cycle, but all visible evidence of a component was lost in the subsequent cycles. Electron micrographs showed only an occasional spheroid particle in the size range of the specific component.

Discussion. The results demonstrate the presence in the plasma of birds with this form of avian leucosis of an abnormal component in amounts great enough to be carried readily through 4 ultracentrifugal cycles. The large number of particles seen in the electron micrographs and their unique morphology permits recognition, unequivocally, of a specific component not present in the plasma of normal or undiseased birds.

There is good reason for supposing that the component is of viral nature, though it may not be a virus of avian leucosis. The resemblance of the particles to some of the bacteriophages(6) and to a lesser extent to

Newcastle disease virus(5) is obvious. Like these viruses, it appears that the particles are probably tailed structures and that the rounded forms represent heads from which the tails were torn during purification. The particles differ greatly from the Newcastle disease virus, however, in size and sedimentation properties(5) and somewhat in appearance. No evidence of disease other than leucosis was seen in the birds. It is not impossible that the component represents some form of bacteriophage; if so, the phage must have been present in the plasma. Cultures from whole blood yielding such plasma have been essentially sterile, and the volume of concentrates and the technique of handling the concentrates would cause doubt of multiplication of bacteriophage in significant amounts during the centrifugal processes. The lack of homogeneity of particle size and shape in electron micrographs and the absence of a sharp boundary in the sedimentation diagram constitute great differences between the characters of this component and analogous properties of the bacteriophages already studied(7-8). Moreover, the high N/DNA ratio indicates a low content of DNA in contrast with the high content of this material in bacteriophages(9,10,11).

In view of its character, source and the conditions under which it was obtained, the component is of much interest whether or not further study indicates it to be the etiological agent of erythro-myeloblastic avian leucosis. As a possible nonviral material, it is unique among the large particles thus far isolated. Should it prove to be a bacteriophage, its association with avian leucosis and its presence in such amounts in plasma would likewise be unique. If the component is a virus not related to avian leucosis, it represents a form hitherto not isolated. Further studies are being made in an attempt to learn the relation of the component to avian leucosis.

Summary. A particulate material has been isolated by ultracentrifugal procedures from the filtered plasma of chickens diseased with avian erythro-myeloblastic leucosis. The particles were either spheroid in shape and of size varying from 60 to 100 mμ diameter or tailed structures with spheroidal heads and tails of 100 to 200 mμ lengths. Preliminary studies on small amounts of material yielded sedimentation diagrams showing definite, though diffuse, boundaries and an approximate sedimentation rate of 630 S. The ratio of nitrogen to desoxyribonucleic acid of the concentrates indicated a low content of the latter. Material of this sort was not found in the plasma of either normal chicks or chicks refractory to infection with the blood or plasma from birds with avian leucosis. The significance of the component in relation to the etiological agent of avian leucosis is discussed.

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Influence of Age, Sex, and Season upon Radioiodine Uptake by the Human Thyroid.* (18256)

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The uptake by the thyroid gland of tracer doses of radioiodine has been extensively investigated as an index of thyroid function (1-5). A wide range of values has been observed in euthyroid subjects, regardless of the tracer technique employed. As a consequence, there is an overlap between the range of uptake found in euthyroid and hyperthyroid or hypothyroid subjects. Any information relative to this overlap might be helpful. Age, sex, and season of the year have been reported as affecting thyroid function(6,7). Nevertheless, a recent review(8) has led to the conclusion that there is little evidence that serum iodine levels are affected by these factors. It has been suggested(6) that tracer uptake may reflect the effect of age without corresponding change in serum iodine. Accordingly, a survey has been made of the tracer tests performed in this laboratory during the past three years, to provide further information about the role of age, sex and season of the year in conditioning the spread of uptake values in euthyroid individuals.

Method. Thyroid uptake of radioiodine has been determined from measurement of activity over thyroid, by a well-shielded Geiger counter in a fixed position 15 cm from the skin over the thyroid isthmus, as described in detail elsewhere(9). Uptake values were established 24 hours after the administration of a tracer dose of I-131, given without carrier. The dosage used for the first six months of the program was 100 μc, and subsequently 40 μc.† A detailed discussion of tracer methods, sources of error, significance of results obtained, and comparison with other tests of thyroid function, has been presented elsewhere(5). The patient material comprises a clinic and in-patient population. Completely accurate information as to diagnosis is available in practically all patients reviewed herein. Values from patients with disorders known to derange the thyroid, whether primary in the gland or secondary to disease elsewhere, have been excluded from this study, as have been values obtained from euthyroid subjects with a history of recent medication with agents known to suppress iodine uptake, such as stable iodine in the form of iodides or gall-bladder dye, thyroid, etc.(5). A few individuals with either hypo- or hyperthyroidism, or treated with the medications just mentioned, have doubtless been included; they are so few as not to invalidate any conclusions drawn, in view of the large series.

Results. Up to May 1, 1950, tracer tests were performed on 1,403 patients; a considerable number of these had more than one test. The total has been reduced to 952 patients (1,050 tests) considered available for

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† The value of the μc is that agreed upon by the New York City Hospital Physicists' Group, and generally called the "New York μc". It is 1.15 times the present Oak Ridge value. That is, 40 New York μc = 46 Oak Ridge μc.

TABLE I. Effect of Age on Average Uptake in Euthyroid Subjects.

Age	No. of cases	Avg	Stand. dev.	Stand. error of mean
<20	49	27.3	10.4	1.43
20-29	195	26.9	7.4	0.53
30-39	228	26.1	8.3	0.55
40-49	191	24.6	8.5	0.61
50-59	150	23.7	8.7	0.71
60-69	88	22.1	8.0	0.85
70-86	51	23.2	9.1	1.28

TABLE II. Effect of Season on Average Uptake in Euthyroid Subjects.

Month	No. of cases	Avg	Stand. dev.	Stand. error of mean
Jan.	101	25.8	8.6	0.86
Feb.	89	25.0	9.4	1.00
Mar.	106	24.0	8.2	0.79
April	100	25.6	9.8	0.98
May	108	24.3	9.8	0.94
June	87	23.8	9.2	0.99
July	62	23.9	8.0	1.02
Aug.	45	23.5	8.5	1.26
Sept.	60	25.3	7.3	0.94
Oct.	98	25.8	7.8	0.79
Nov.	99	25.5	8.7	0.87
Dec.	95	25.2	8.8	0.90

the present study for the reasons mentioned under "Methods." Of the 1,050 tests thus analyzed, 989 fell into the 10-40% range of uptake of I-131 at 24 hours, 20 in the less than 10%, and 41 in the 40-51% range. No uptake value in excess of 51% at 24 hours was observed in the euthyroid group. Information as to time of year is available for all tests (Table II). Results of measurement for every month over a 3-year period are available. Age and sex are known for 952 of the 1,050 tests (Table III). There was a ratio of males to females of 1 to 2.3. Ages ranged from 16 to 86.

Effect of Sex. In the entire data there were 284 tests in men and 668 in females for which adequate sex and age information was available (Tables I and III). The overall uptake average for the former was 23.5% at 24 hours, and for the latter 25.8% (Table III). The standard error of the mean was 0.42 and 0.31, respectively. The slightly higher uptake for women over men is still seen if the data are rearranged to express both decades of age and sex. The observed difference in uptake, while statistically significant for these large series, is not of a degree to be clinically significant.

Effect of age. Results of analysis by age decades (both sexes together) are shown in Table I. The progressive decrease toward old age is statistically significant, although probably not enough to be clinically important. The slight but steady decrease in uptake with each decade of advancing age is also seen when the data are broken down according to both decades and sex.

Seasonal Variation. Average monthly uptakes (both sexes and all ages) are given in Table II. The apparent decrease toward midsummer is not statistically significant.

Factors Considered Separately. In order to make sure that, in studying any one of the 3 factors under discussion, variations due to the other 2 were not introducing errors, the entire set of data was tabulated to show variations for sex and age, winter and summer. (Winter was arbitrarily set as November to April, inclusive; Summer as May to October). This is shown in Table III. The separate factors may now be examined independently. Considering men and women in winter, in the different age groups, it is seen that the average uptake by women is always a little higher. The difference is not so evident in summer. However, many of the subgroups are so small that sampling fluctuations may be large. A detailed statistical analysis has not been deemed necessary. Similarly, the trend toward lower values in old age is not so evident when the sub-groups are examined.

A further check on possible influence of seasonal variation was found in examining the tests which had been repeated in a small group of the individuals without thyroid dysfunction. There were 38 subjects on whom two or more tests had been performed at different seasons. If seasons are divided into the usual 4—winter: Dec., Jan., Feb.; spring: Mar., Apr., May; summer: June, July, August; autumn: Sept., Oct., Nov., the findings were as follows: 16 patients, no change from season to season.‡ 10 patients,

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‡ By "no change" is meant a shift of less than 10% of a value, *e.g.*, a shift from 28 to 30% uptake.

TABLE III. Analysis of Euthyroid Data by Age, Sex, and Season.

	Men						Women					
	Winter			Summer			Winter			Summer		
Age	No.	Avg	Stand. dev.	No.	Avg	Stand. dev.	No.	Avg	Stand. dev.	No.	Avg	Stand. dev.
<20	9	27.9	9.2	8	26.1	10.0	20	27.8	12.2	12	26.8	9.2
20-29	19	24.5	6.7	11	30.3	5.7	77	28.0	7.9	88	26.2	7.7
30-39	28	24.7	8.5	14	23.4	7.6	111	27.5	8.3	75	25.1	8.5
40-49	52	22.5	7.8	21	24.0	8.6	67	26.0	9.3	51	25.2	8.4
50-59	41	23.4	6.0	25	21.7	7.2	51	25.2	9.5	33	23.3	10.8
60-69	21	21.3	7.6	13	21.5	6.6	32	23.2	8.5	22	21.7	8.4
70-86	16	20.9	7.6	6	27.8	6.9	17	22.7	7.8	12	24.6	14

Totals	No.	Avg	Stand. dev.	Stand. error of means
All men	284	23.5	7.6	0.42
All women	668	25.8	8.5	0.31
All winter	561	25.4	8.4	0.35
All summer	391	24.8	8.4	0.43

lower reading in warmer season. 6 patients, lower reading in colder season. 6 patients, indeterminate. (In each of these, there were 3 tests, and shifts occurred in both directions). Accordingly, the results in this small group with repeated determinations are in accord with the statistical findings of the entire series, in that no significant seasonal variation was found.

Discussion and Conclusions. The uptake of radioiodine by the normal thyroid is a reflection, in general, of the level of secretory activity. This relationship may be disrupted by a number of influences not usually present in an unselected population, such as the administration of stable iodine or anti-thyroid drugs. In the present study, such extraneous influences have been excluded, granting an inevitable small error. Three findings emerge from the data. The level of thyroid activity in the euthyroid individual as determined from the radioactivity of the thyroid gland 24 hours after a dose of 40 to 100 μc I-131 is unaffected by season of the year. Uptake appears to decrease progressively, though slightly, with age. Uptake is a little higher in women than in men at all ages.

Perlmutter and Riggs(6) have called attention to a progressive decrease with age in rate of radioiodine uptake early after administration, of a very marked degree. They employed the "accumulation gradient" as a criterion, determined during the first four hours after the ingestion of I-131. In a limited sampling of their patients tested chemically for serum precipitable iodine, as well as with radioiodine, they found no corresponding variation in the serum iodine levels. This led the authors to postulate a decreasing utilization of thyroid hormone at the periphery, with advancing age, rather than any intrinsic diminution in thyroid activity.

The findings of the present study also show a stepwise decline in the uptake of I-131 as measured at 24 hours, but of an entirely different range of magnitude than that reported for the "accumulation gradient." It is generally agreed that the 24 hour determination is a more reliable index of overall thyroid function than the gradient which may be influenced by absorptive and circulatory factors. Since the degree of decrease in 24-hour uptake is only questionably significant clinically, unlike the decline in gradient with advancing age, it is likely that a corresponding change in serum precipitable iodine level might be concealed by a small sampling in euthyroid subjects. Thus a far larger series of patients would have to be studied in this regard than was examined by Perlmutter and Riggs. It therefore still appears to be open to question whether thyroid function decreases gradually with age, whether peripheral utilization of thyroid hormone is diminished with age, or whether the slight degree of decline in radioiodine uptake represents some extraneous mechanism which affects the rate of iodine accumulation but which does not influence the overall secretory activity of the gland.

The persistently higher radioiodine uptake by women over men raises the same question of biological versus statistical significance. The higher uptake by women does not correspond with the lower basal metabolic rate found in this sex compared to men of the same age. No ready explanation for the uptake difference is at hand, but the data serve to emphasize that other factors influence basal metabolic rate(10) besides the level of thyroid hormone secretion.

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Summary. 1. The percentage of a tracer dose of radioactive iodine deposited in the thyroid gland at the end of 24 hours was determined by measurement of radio-activity directly over the gland in 1,050 tests in 1952 apparently euthyroid individuals. 2. It is concluded that, within the New York area, there is statistically no effect of season, on the average uptake of radioiodine by the thyroid gland of the euthyroid individual. 3. The 24-hour uptake of radioiodine decreases progressively but slightly with each decade of age. The extent of fall is statistically, but not clinically, significant. 4. Women in all age groups show a slightly higher average uptake than men. The difference is not clinically significant.

Appreciation is expressed to Dr. John Fertig of the School of Public Health, Columbia University for reviewing the statistical material.

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Protective Action of N-Allyl-Normorphine Against Respiratory Depression Produced by Some Compounds Related to Morphine.*† (18257)

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Some of the pharmacological properties of N-allyl-normorphine have previously been investigated by Unna(1) and Hart and McCawley(2). These studies demonstrated that N-allyl-normorphine prevents, or abolishes, certain actions of morphine, *i.e.*, the respiratory depression, the excitatory action in cats, and increased the lethal dose. No information seems to be available showing whether the protection of the respiratory center afforded by N-allyl-normorphine against morphine extends to other narcotics, therefore, data on this point were collected for the following: codeine (methylmorphine), Dilaudid (dihydromorphinone), Demerol (ethyl l-methyl-4-carboxylate-hydrochloride), Metopon (methyl-dihydromorphine), and Methadon (6-dimethylamino-4, 4-diphenyl-3-heptanone hydrochloride).

Methods. The dogs were anesthetized with sodium barbital 250 mg/kg and a sound passed down the left carotid into the aorta for the registration of blood pressure with a Hamilton manometer(3). Respiratory rate was recorded by attaching a chest plethysmograph to a Hamilton manometer. Two pro-

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† We wish to express our appreciation to the following for making certain drugs available: Dr. George A. Emerson, University of Texas Medical Branch, Galveston, Texas, N-allyl-normorphine, Dr. Karl H. Beyer, Sharp & Dohme, Metopon, and Mr. Kenneth M. Smoot, Winthrop-Stearns, Inc., Methadon.

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TABLE I. Lethal Doses of Various Narcotics in Dogs.

No. of dogs	Drug	Dose, mg/kg	No. dogs dying of respiratory failure	Time until death, min.
	Sodium barbital, 250 mg/kg			
6	Demerol	10	4	2 to 5
3	”	15	3	
6	Methadon	2.5	4	1 to 2
3	”	3.0	3	
3	Metopon	0.5	1	1 to 2
2	”	1.5	2	
2	”	2.5	2	
4	Dilaudid	2.0	4	1 to 2
2	Codeine	15	1	2 to 5
6	”	20	6	
	Chloralose, 120 mg/kg			
6	Methadon	2.5	6	30 to 60

TABLE II. Effect of N-Allyl-Normorphine on Respiration in Dogs Receiving Lethal Doses of Various Narcotics.

No. of dogs	Drug	Dose	No. dogs dying of respiratory failure
	N-allyl-normorphine, 20 mg/kg, administered after narcotic.		
4	Demerol	10	3
2	”	15	All
2	”	30	”
3	Methadon	2.5	0
3	”	5	0
3	Metopon	2	0
3	”	4	0
3	Dilaudid	2.5	0
3	”	5	0*
3	Codeine	20	0
3	”	40	0
	N-allyl-normorphine, 20 mg/kg, administered before narcotic.		
6	Demerol	10	5
4	Methadon	2.5	0
6	Metopon	2	0
6	Dilaudid	2.5	0
6	Codeine	40	0

* 2 required additional amounts after 30-60 min.

cedures were used in this study. One, the N-allyl-normorphine 20 mg/kg was given i.v. and 20 minutes later the lethal dose of one of the narcotics was given. The other, was to give a lethal dose of a narcotic and after respiration had stopped for approximately 30 seconds to administer the N-allyl-normorphine. In addition to the lethal dose, twice that amount was also tried for each drug.

Results. N-allyl-normorphine 20 mg/kg may have a transient effect on respiration and circulation. The respiratory rate either remains unchanged or there may be an increase for 1 or 2 minutes. There is usually a slight fall in blood pressure but it returns to the control level within 5 to 15 minutes.

It was necessary to determine, at least approximately, the lethal amounts of the various narcotics used under the conditions of these experiments. These data are included in Table I. In 6 dogs in which Methadon was used chloralose was substituted for the sodium barbital to test whether there might be a significant difference in the lethal dose as a result of a different anesthetic. The use of chloralose instead of barbital did not effect the lethal dose but respiratory arrest when chloralose was used did not occur for 30 to 60 minutes after injection. Respiration during the interval after injection until respiratory arrest was in the majority of instances of the Cheyne-Stokes type but in 2 dogs Biot's breathing occurred.

When N-allyl-normorphine, Table II, was injected either before or 30 to 45 seconds after lethal amounts of the other drugs there was no evidence of a significant decrease in either rate or depth of respiration except with Demerol. N-allyl-normorphine did not protect against a lethal dose of Demerol.

Discussion. The antagonistic action which N-allyl-normorphine has on the respiratory depression produced by morphine has been shown to extend to certain morphine derivatives and synthetic substances of related structure, with the exception of Demerol. The structural formulae for Demerol and Methadon can be written to simulate the morphine structure but there is some doubt whether this is warranted. However, since N-allyl-normorphine antagonizes the respiratory depression produced by Methadon but not that of Demerol, it tends to support the validity of considering Methadon to be related to morphine in structure.

Conclusions. 1. N-allyl-normorphine prevents or antagonizes the respiratory depression produced by codeine, Dilaudid, Metopon and Methadon. 2. It did not provide protection against the respiratory depression caused by Demerol.

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Enhancement of Myotrophic and Androgenic Responses by Aqueous Suspension of Steroids.* (18258)

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Estrogens(1-4), progesterone(2), desoxycorticosterone(5) and androgens(6) have been reported to be more effective when administered as aqueous suspensions than in oil solution. In this study a comparison of the effect of oil solution with aqueous suspension of certain androgens on the temporal muscle and accessory sex organs of the guinea pig(7) has been made.

Procedure. The guinea pigs were of the Hartley strain (Tumblebrook Farms) and were obtained at 325-375 g body weight(8). They were kept in individual cages and fed Rockland guinea pig diet containing vitamin C, *ad libitum.* Once per week each animal was given 12.5 mg vitamin C in a tablet by mouth. When the guinea pigs reached 395-405 g body weight they were castrated. Thirty to 35 days later the androgens‡ were injected subcutaneously at approximately 5 p.m. on each day for 14 days. The guinea pigs were weighed 3 times per week beginning at arrival and also at autopsy. Any animal that did not show a good growth response was discarded. On the morning of the 15th day the animals were killed and the accessory sex organs and the temporal muscles were removed and weighed on a Roller-Smith torsion balance.

Results. Body Weight. All of the androgens except testosterone in oil solution at 0.25 mg/day produced a greater increase in body weight than was shown by the control animals.

Myotrophic and Androgenic Effects. At both dose levels the aqueous suspension of testosterone produced a much greater increase in the weight of temporal muscles and seminal vesicles and prostates than the oil solution did (Table I); the aqueous suspension enhanced the myotrophic effect roughly 3 times and the androgenic 9-16 times depending on the dose. Furthermore, the aqueous suspension of testosterone was only slightly less effective than testosterone propionate in oil solution.

An aqueous suspension of androstanediol-3 α, 17 α had a slightly smaller androgenic effect but a greater myotrophic effect than the aqueous suspension of testosterone.

Discussion. The increase in body weight produced by the androgens is in agreement with previous observations after implantation of pellets of androgens(7).

The greater efficacy of the aqueous suspension of the androgens on the temporal muscle of the guinea pig suggests this method of administration as suitable for stimulation of protein anabolic processes.

The greater efficacy of the aqueous suspension of androstanediol-3 α, 17 α as compared to testosterone on the temporal muscle

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* This investigation was supported by a grant from Ayerst, McKenna and Harrison Ltd.

† Present address: Oklahoma Medical Research Institute, Oklahoma Medical Center, Oklahoma City, Oklahoma.

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‡ The testosterone propionate (Perandren) testosterone, and the androstanediol-3 α, 17 α ("micro-crystalline" aqueous suspension) were provided by Ciba Pharmaceutical Products, Inc. and the testosterone ("micro-crystalline" aqueous suspension) by Ayerst, McKenna, Harrison and Co.

TABLE I. Enhancement of Myotrophic and Androgenic Responses by Aqueous Suspension of Steroids.

Treatment		Guinea pigs	Dose,	Body wt		Increase		
Steroid	Vehicle	No.	mg/day	Initial, g	Increase, g	Temporal muscles (M) mg*	Sem. ves. + pros. (A) mg*	M/A†
Control		14		539	54	(690)	(550)	
Testosterone	Oil	4	.25	535	56	83	129	
	Susp.	4	.25	564	86	184	1072	.17
Androstanediol-3 α, 17 α	”	5	.25	551	79	256	960	.27
Testosterone	Oil	4	.50	539	85	131	114	1.15
	Susp.	4	.50	584	79	426	1756	.24
Test. propionate	Oil	4	.50	574	77	456	2230	.21
Androstanediol-3 α, 17 α	Susp.	4	.50	573	85	452	1560	.29

* The values are the increases above the control values which are in parentheses.

† M/A = The growth of the temporal muscles divided by the growth of the seminal vesicles and prostates.

plus its smaller effect on the accessory sex organs in agreement with pellet studies(7) now makes it possible to carry out clinical studies with this interesting steroid. The extremely low solubility of androstanediol-3 α, 17 α in oil(9) has previously made such studies difficult.

Summary. An aqueous microcrystalline suspension of testosterone was 3 times more effective in stimulating the growth of the temporal muscles and 9-16 times more effective on the accessory sex organs than an olive oil solution of testosterone and as effective as a sesame oil solution of testosterone propionate. An aqueous microcrystalline suspension of androstanediol-3 α, 17 α was slightly more effective on the temporal muscle and less effective on the accessory sex organs than a similar suspension of testosterone.

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Protein Anabolic Activity of Pregnant Mares' Urine.* (18259)

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The presence of a protein anabolic factor(s) in androgenic male urine extracts was reported(1) 15 years ago. A similar factor(s) now has been found in pregnant mares' urine.

Procedure. Pregnant mares' urine was extracted† by the method used for human male urine(1) with minor modifications. The neutral extracts were fractionated‡ by separation into ketonic and non-ketonic portions by means of Girard's T reagent and were then further fractionated by chromatography with magnesium silicate as adsorbent(2). The

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* This investigation was supported by a grant from Ayerst, McKenna and Harrison Ltd

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† The neutral extracts were prepared by the Ayerst, McKenna and Harrison Ltd.

‡ The following phase of the program was carried out at Rochester with the assistance of Betty Beall.

TABLE I. Characteristics and Solubility in Olive Oil of Protein Anabolic Fractions of Pregnant Mares' Urine.

Fraction	Eluant	Wt, g	Physical characteristics	Solubility in olive oil
	Total ketonic.			
LH-62-1	(α-ketonic)	0.95	Fluid, dark red oil	Slight ppt.
	Ketonic fractions.			
C-3-17-I	Benzene to benzene: ether (97, 95, 90, 75)*	4.94	Taffy-like	No ppt.
II	Benzene: ether (75, 50)* Ether, ether: methyl alcohol (90)	1.55	Brittle solid	Slight ppt.
III	Ether: methyl alcohol (90, 50) Methyl alcohol	2.27	Powder	Copious dark red ppt.

* The figures in the parentheses indicate the percent of the first solvent in the respective mixtures.

effectiveness of these procedures was determined by the Zimmermann(3), Pincus(4) and 2,4 dinitrophenylhydrazine color tests and androgenic(5), estrogenic (6) and protein anabolic(7) assays in the rat and the myotrophic-androgenic test in the guinea pig(8). Since the results indicated the presence of the protein anabolic factor, larger amounts were prepared and the activity was finally established in the dog. The 2 ketonic fractions studied in the dog were prepared as follows[§]. A total ketonic fraction, 15 g, was chromatographed to give the following 4 eluates: with carbon tetrachloride 1.753 g, benzene 1.640 g, benzene-methyl alcohol (95:5) 10.946 g, and methyl alcohol 0.401 g. The third and largest fraction was rechromatographed and the eluates pooled into 4 fractions (Table I). In addition an α-ketonic fraction (Table I) was prepared from another large neutral extract[¶]. The fractions were dissolved in acetone and sufficient olive oil was added to give a concentration of 100 mg/ml but some of the fractions contained material which was insoluble in the oil (Table I).

The metabolism studies[‖] were carried out essentially as described earlier(1,9,10). Dog 68 was a dark brown, short-haired mongrel probably of terrier and hound lineage. Dog 72 was a white short-haired mongrel of predominantly bull and terrier lineage. The dogs were castrated and maintained in individual metal metabolism cages. They were fed a diet consisting of beef heart 52, cracker meal 22, hydrogenated vegetable oil 5.2, cod liver oil 1.3, dry brewers' yeast (Fleischmann's No. 2019) 1.3, cellu flour 1.3, and Wesson's salt mixture 1.3. The amount fed was estimated by the formula of Cowgill and Drabkin(11) but small changes had to be made to establish and maintain constant body weight and nitrogen equilibrium. The urine collections were made under toluene and the dogs were catherized at 2 day intervals, occasionally one or 3 day intervals. The first

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[§] These preparations were made by Ayerst, McKenna and Harrison Ltd. in accordance with the results of the extensive exploratory studies at Rochester.

[¶] This fraction was prepared by Dr. K. Dobriner of the Sloan-Kettering Institute.

[‖] William van de Mark and Arthur Sakamoto assisted in these studies.

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TABLE II. Protein Anabolic Factor(s) of Pregnant Mares' Urine.

Treatment*	Dose mg/day	Dose days	Max. N retained, g/day	Incr. body wt, kg
	Dog 68†			
Testosterone§	20	13	1.32	.45
	100	2		
α-ketonic, LH 62-1	150	5	1.40	.45
	Dog 72‡			
Testosterone§	20	13	1.03	.36
	100	5		
Ketonic, C-3-17-II	200	5	0.62	.36
Ketonic, C-3-17-III	200	8	0.90	.45

* First exp. 120 days after castration.
† Body wt, 9.3 kg; N intake, 5.40 g/day.
‡ Body wt, 11.2 kg; N intake, 6.60 g/day.
§ Aqueous micro-crystalline suspension at 100 mg/ml.

experiment was not begun until 120 days after castration. The dietary nitrogen was determined by the macro-Kjeldahl procedure with distillation of aliquots by the micro procedure. The urine nitrogen was determined by the micro-Kjeldahl method.

Results. The testosterone and the urine fractions (Table II) gave the typical nitrogen retention and body weight responses (1, 9,10). The urinary nitrogen excretion promptly decreased to a minimum where it was maintained until cessation of injections when an extra excretion of nitrogen occurred. The body weight increased with the retention of nitrogen and decreased slightly on cessation of injections. Table II shows the maximum nitrogen retained (average of several periods) and the maximum increase in body weight.

Testosterone at 20 mg per day in a micro-crystalline suspension produced a very marked drop in urinary nitrogen and an increase in body weight. The smaller dog interestingly responded to a greater degree than the slightly larger dog. The total ketonic fraction at 5 to 7.5 times the dose of testosterone produced effects identical with those of the testosterone as did the 2 ketonic fractions at 10 times the dose of testosterone. The first ketonic fraction (C-3-17-1) showed no protein anabolic properties in the rat but this fraction was not studied in the dog.

Discussion. The establishment of protein anabolic activity in the ketonic fraction of extracts of pregnant mares' urine does not exclude the presence of similar properties in other fractions. Furthermore, the activity in the ketonic materials may be due to more than one compound since the 2 chromatographic fractions exhibited a high degree of activity. Most of the activity probably is in the α-steroid(s). Many ketonic and also non-ketonic steroids now are known to stimulate growth(7,8,12,13).

The relative potency of these extracts cannot be assessed until the minimum effective dosages are determined. Furthermore, the testosterone was not studied in oil solution. The micro-crystalline suspension was arbitrarily chosen in order to compare it later with an oil solution. A rough quantitative comparison can be made between the urine extracts and the testosterone. The micro-crystalline suspension is 3 times as effective in the myotrophic and 9-16 times as effective in the androgenic test in the guinea pig as an oil solution of testosterone(14). On this basis, the ketonic fractions may be considered to possess at least one half the potency of testosterone. Furthermore, the maximal responses were identical or very similar to those produced by testosterone. Therefore, the active compounds must be considered of relatively high potency.

Summary. Pregnant mares' urine has been demonstrated to contain a relatively high protein anabolic activity as determined by nitrogen balance and body weight studies in castrated dogs. A large proportion of the activity is in the α-ketonic fraction of a neutral fat-soluble extract and is probably due to more than one compound.

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Effect of Dietary Protein Restriction on Plasma Volume, "Available (Thiocyanate) Volume" and Muscle Water. (18260)

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In the past, the majority of workers have supported the theory that the production of "malnutritional edema" is closely associated with hypoproteinemia. On the other hand, various more recent authors have doubted the importance of hypoproteinemia in this respect. Thus, the results of the experiments by Weech, Snelling and Goettsch(1) and by McClure and Hinman(2) were taken to give strong support to the view that a correlation exists between the fall of the plasma protein concentration and the accumulation of edema fluid. In contrast, Henschel, Mickelsen, Taylor and Keys(3) concluded that though hypoproteinemia may play a role, "the edema in simple caloric starvation is largely a reflection of a reduction in cellular mass without large change in the absolute amount of extracellular fluid."

The present experiments were carried out on rats and dogs maintained on protein-deficient diets. The study was undertaken to determine whether changes of the "available (thiocyanate) volume" ("SCN") and of the skeletal muscle chloride space held any relation to each other and to the extent of the plasma protein deficit.

Methods. In the experiments on rats, young adult females, weighing between 200 and 258 g, were placed on a control diet(4) or on a diet containing fresh raw carrots as the only source of protein. Vitamin supplementation was made orally as in previous experiments(4). Rats on the carrot diet were sacrificed at the end of 2 weeks, and at the end of succeeding 2-week periods through 14 weeks. In the experiments on dogs, young adult animals were placed on a constant diet(5) for a period of at least 7 days before the experiments were undertaken. The majority of the dogs received for 3 weeks a protein-free diet, differing from the control diet only in glucose substitution for the casein. In the case of 4 dogs the dietary period was extended to 74 days. Plasma volume (P.V.) and "SCN" were determined on dogs by the direct method of Gregersen and Stewart(6) as adapted to the photoelectric colorimeter by Gibson and Evelyn(7). The method was modified slightly when applied to rats. The collection, sampling and chemical analyses of the blood and skeletal muscle were carried out as previously described(4,5). The extracellular and intracellular water partition of skeletal muscle was calculated in the manner outlined by Hastings and Eichelberger(8).

Results and discussion. Plasma volume and "available (thiocyanate) volume."* From the data presented in Table I it is seen that in rats with plasma albumin deficit the P.V. tended to be lowered when expressed per unit of control weight, and tended to be increased when calculated per unit of experimental

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* None of the dogs receiving the protein-free diet for the 3-week period exhibited clinical edema, while one of the 4 dogs given the diet for 74 days showed a small amount of peritoneal fluid when the experiment was terminated. Of 12 rats placed on the carrot diet for 10 weeks or longer, 6 exhibited some fluid in the peritoneal cavity when they were sacrificed.

TABLE I. Average Plasma Albumin and ‘‘Fluid’’ Volume Changes in Rats and Dogs Receiving Protein-Deficient Diets.*

		Plasma vol. as ml		‘‘SCN’’ vol. as ml		Ratio:	Skeletal muscle per kg fat-free tissue			
Body wt†	Plasma albumin, g/100 ml	Per control wt§	Per exper. wt§	Per control wt§	Per exper. wt§	‘‘SCN’’ / P.V.	H_2O g	Cl mEq	Extracell. H_2O as g	Intracell. H_2O as g‖
				Rats receiving carrot diet.						
222(15)	2.81	4.08		26.3		6.5	762.4(6)	10.5	95	738
178(2)	2.56	3.55	4.33	24.3	30.0	6.9	765.5	12.3	113	736
162(7)	2.38	3.18	4.58	20.3	29.1	6.4	771.0	14.2	130	738
142(5)	2.07	2.83	4.80	17.4	29.5	6.2	770.1	19.2	172	724
143(9)	1.89	3.30	5.34	19.4	31.4	5.9	773.5	18.8	175	727
131(3)	1.65	3.57	6.06	20.7	35.0	5.8	784.3	28.0	244	718
				Dogs receiving protein-free diet.						
11.8(82)	3.40	47.1		293		6.3	758.7(5)	11.7	96	733
9.4(5)	2.85	40.0	42.4	266	282	6.7				
9.1(4)	2.62	39.2	41.5	255	272	6.5				
8.9(6)	2.33	41.8	45.7	278	303	6.7				
9.6(7)	2.08	42.0	46.6	305	338	7.3				
10.1(3)	1.96	44.6	47.8	347	371	7.8				
9.0(4)‡	1.86	32.5	51.8	215	342	6.7	776.8	18.2	144	741

* Data represent averages grouped according to extent of plasma albumin deficit.

† Exp. weight, given as g for rats and as kg for dogs.

‡ Maintained on diet for 74 days and data tabulated separately.

§ Per 100 g in the case of rats and per kg in the case of dogs.

‖ Per 1000 g of muscle cells.

weight. The "SCN" in these animals was found to be changed in a similar manner and consequently the ratio of "SCN": P.V. was not increased. In general, similar findings were obtained in the dogs placed on the protein-free diet. However, the ratio of "SCN": P.V. tended to be increased in those dogs given the diet for 3 weeks and exhibiting a marked reduction of plasma albumin. On the other hand, the average ratio of "SCN" : P.V. was not appreciably different from the control value in those dogs placed on the protein-free diet for 74 days despite an even greater reduction of the plasma albumin concentration.

The results of the present experiments are quite comparable to those obtained in a study(3) of normal men subsisting on an European type of famine diet for an extended period of time. The "SCN" calculated per unit of experimental weight indicated the presence of a relative excess of extracellular fluid even though there was a decrease in the total "SCN."

Skeletal muscle water and chloride space. From the average data presented in Table I, it will be seen that in rats the muscle water and chloride contents progressively increased as the plasma albumin concentration was reduced. The extracellular water content calculated on the basis of muscle chloride was increased while the intracellular water per 1000 g muscle cells remained relatively unchanged. Similar changes were observed in the skeletal muscle of the 4 dogs placed on the protein-free diet for 74 days.

The relation between the extracellular water content of muscle and the plasma albumin deficit noted here is similar to that observed by McClure and Hinman(2) who used muscle sodium as the basis for calculation of the extracellular phase. Recently, in rats receiving a protein-deficient diet or undergoing starvation, Dicker(9,10) observed an increase of the skeletal muscle chloride space which could be detected in spite of a normal plasma protein concentration. When considered by itself the increase in the extracellular water of muscle calculated from muscle chloride (or sodium) can be interpreted to indicate an accumulation of total extracellular water. However, the simultaneous determination of the "SCN" revealed a loss or a tendency toward a stabilization, rather than an increase, of total extracellular fluid in animals placed

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on a protein-deficient diet and exhibiting hypoproteinemia. Such a situation can be interpreted along the lines expressed by Henschel, Mickelsen, Taylor and Keys(3) as representing a reduction of cellular mass without a large change in the absolute amount of extracellular fluid.

Summary. Hypoproteinemia induced in rats and dogs by protein-deficient diets was accompanied by a fall of the P.V. and "SCN" when expressed per unit of control weight and increased when expressed per unit of experimental weight. Thus, there was a decrease in the absolute extracellular volume, as measured by thiocyanate, in the presence of a "relative" extracellular hydration. The extracellular phase of muscle calculated on the basis of the muscle chloride content progressively increased as the plasma albumin decreased. This can be interpreted as indicating an accumulation of extracellular fluid accompanying a progressive hypoproteinemia. However, because of the observed decrease of the "SCN," these observations appear better explained on the basis of a reduction in cellular mass without a large change in the absolute amount of extracellular fluid.

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Protective Action of Antibiotics Against the Toxin of *Pasteurella pestis* in Mice. (18261)

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Many investigators(1-4) have pointed out the antitoxic activity of penicillin and the sulfonamides against various bacterial toxins. Both penicillin and aureomycin exhibit antitoxic activity against certain strains of the psittacosis-lymphogranuloma venereum microparasites(5) and aureomycin, against some viral and rickettsial toxins(6). Penicillin and the sulfonamides, however, offer little or no protection against toxins of *Pasteurella pestis*(7). Because more success in experimental therapy has been achieved with other antibiotics(8,9), the following experimental work was instigated.

Exp. 1. *Protection with Aureomycin, Terramycin and Chloramphenicol.* The toxins were obtained by extracting a 2% suspension of acetone-killed and dried, agar-grown *Pasteurella pestis* (Yreka) in physiologic saline. The bacteria were removed by centrifugation. To test the protective value of aureomycin, terramycin and chloramphenicol, 1.0 ml of a solution containing 2 mg/ml of the antibiotic was injected intraperitoneally into albino (Swiss) mice. Four hours later, 0.5 ml of a serial dilution of the toxic solution was injected intravenously into groups of 10 mice each. A control group of mice was given 1.0 ml of physiologic saline. Results are presented in Table I.

Exp. 2. *Duration of protection.* In this series, the period between giving the antibiotic and giving the toxin was varied to determine how long the protection lasts. The same amount of the drug was given in each dose by the same route; 1, 12 and/or 24 hours later, plague toxin (2 LD_{50}) was given intra-

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TABLE I. Titration of Plague Toxin in Mice Pretreated with Antibiotics.

Antibiotic* (2 mg/ml, i.p.)	Survivors/total mice, 48 hr after toxin inj. Toxin dilution (0.5 ml, i.v.) 1:40	1:80	1:160	1:320	1:640	1:1280
Aureomycin	0/10	1/10	8/10	9/10		
Terramycin	1/10	5/10	10/10	10/10	10/10	
Chloramphenicol		3/10	3/10	8/10	9/10	
Controls		0/10	1/10	1/10	6/10	10/10

* Given 4 hr before toxin. Controls were given physiologic sodium chloride sol.

TABLE II. Duration of Protection of Mice Against *P. pestis* Toxin.*

Antibiotics (2 mg/ml, i.p.)	Hr before toxin inj.	No. mice	Survival rate 8-12 hr	20-24 hr	48 hr
Aureomycin	24	20	10	10	10
	12	20	95	95	95
	1	20	90	75	75
	12, 6, 1	20	100	100	100
Terramycin	12	10	80	70	70
	1	10	80	80	80
Chloramphenicol	12, 6, 1	20	70	65	60
	1	20	60	35	35
Streptomycin	12, 6, 1	20	50	40	40
	1	20	50	35	25
Controls		20	35	35	25

* 2 LD_{50} of plague toxin given i.v.

venously. The results are presented in Table II. The protective action of aureomycin and terramycin lasted at least 12, but not 24 hours. Only when 6 mg of chloramphenicol was given did the survival rate reflect protection. Streptomycin, the most effective therapeutically, exerted no antitoxic effect, even when 6.0 mg was given.

Summary. Intraperitoneal administration of aureomycin, chloramphenicol and terramycin in 2.0 mg doses within 12 hours of intravenous injection of plague toxin conferred definite, but limited, antitoxic protection to mice. Streptomycin was without this protective action. Aureomycin injected 1 hour or 12 hours prior to toxin exerted the greatest protective effect. Chloramphenicol was not as effective as either aureomycin or terramycin against *Pasteurella pestis* toxin.

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Interference of Aureomycin and of Terramycin with Action of Penicillin *in Vitro*.* (18262)

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Recent studies have established that one antibiotic may interfere with the action of another both *in vitro* and *in vivo*(1-6). It has been shown that chloramphenicol antagonizes penicillin in the test tube and in a streptococcal infection of mice(3,4,6). The interference *in vitro* of aureomycin with the bactericidal action of penicillin against enterococci has also been demonstrated(6).

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* Supported in part by a grant from the Research Committee of the University of California School of Medicine.

† With the technical assistance of M. J. Bruff and E. Jahn.

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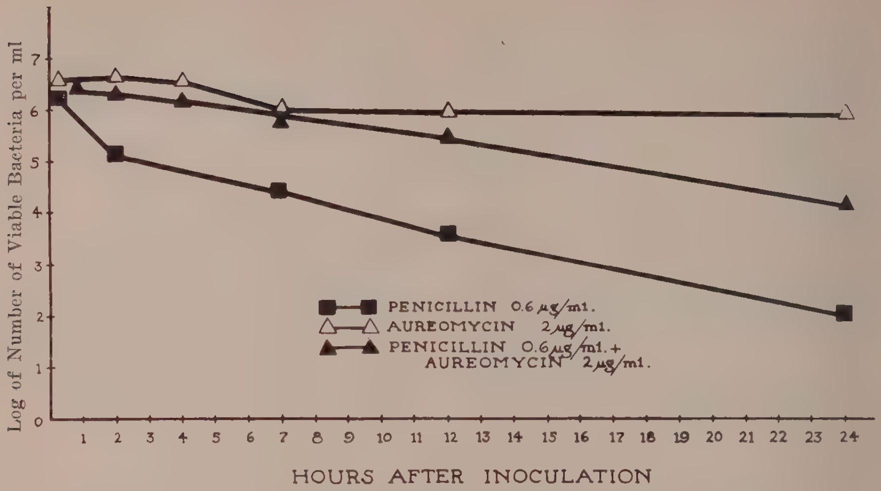

FIG. 1.
Interference of aureomycin with action of penicillin on *Streptococcus pyogenes.*

Materials and methods. In the present study, *Streptococcus pyogenes* (C203)[‡] and *Klebsiella pneumoniae* (A-D)[§] were used as test organisms. Crystalline potassium penicillin G, aureomycin hydrochloride (No. 7-6116)[‖], and terramycin hydrochloride[¶] were dissolved in 0.85% sodium chloride solution to make a concentration of 2.5 mg/ml. The solutions of aureomycin and of terramycin were sterilized by Seitz filtration. The aureomycin solution was quickly frozen and stored at –20°C. Final dilutions of the antibiotics freshly prepared in broth in a volume of 19 ml were inoculated with 1 ml of an 18-hour broth culture containing 10^7-10^8 bacteria per ml. Samples of 0.5 ml were removed at intervals during incubation at 37°C and the number of viable bacteria was estimated by plate counts. The culture media used were Proteose Peptone No. 3 agar (Difco), to which 2% blood and 4% penicillinase solution (Bacto-Penase) were added for tests with *S. pyogenes,* and broth with the same base.

Results. Both aureomycin and terramycin interfered with the bactericidal action of penicillin against *S. pyogenes* and *K. pneumoniae.* The bactericidal rate of penicillin alone was reduced by the addition of certain concentrations of either aureomycin or terramycin. Representative experimental results are shown in Fig. 1 and 2.

Against *S. pyogenes,* penicillin was highly active, 0.006 µg/ml was rapidly bactericidal; aureomycin and terramycin were less active, 1-10 µg/ml were mainly bacteriostatic. When either of the less active drugs was added to penicillin the rate of death of the streptococci approximated that obtained with the less effective antibiotic alone (Fig. 1). Increase in the concentration of aureomycin or terramycin up to 10-50 µg/ml resulted in a slight increase in their antagonism toward penicillin. When the concentration of aureomycin was further increased to the point where it was moderately bactericidal by itself interference with penicillin action could no longer be demonstrated.

Against *K. pneumoniae,* each of the 3 antibiotics was moderately active in about equal

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[‡] Originally obtained from Dr. H. Eagle, Natl. Inst. of Health, Bethesda, Md.

[§] Originally obtained from Dr. E. R. Jackson, American Cyanamid Co., Stamford, Conn.

[‖] Kindly supplied by Dr. S. H. Hardy, Lederle Laboratories, Pearl River, N. Y.

[¶] Kindly supplied by Dr. H. A. Anderson, University of California School of Medicine (Lot WBW 507019).

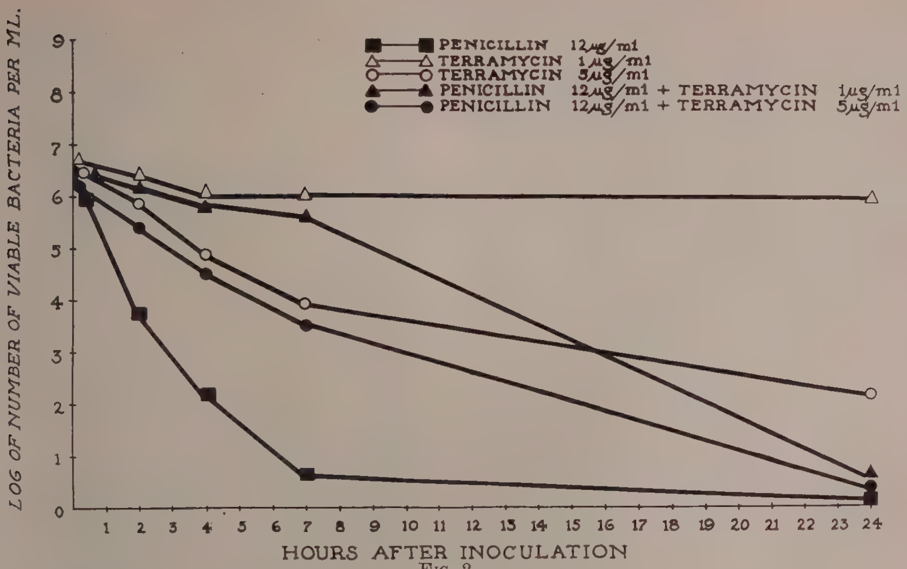

Fig. 2.

Interference of terramycin with action of penicillin on *Klebsiella pneumoniae.* Effect of varying concentrations of terramycin.

degree; 5-10 µg/ml usually killed 99.9% of the inoculated bacteria within 24 hours. Mixtures of 10 µg/ml of aureomycin or terramycin with 0.6-6 µg/ml of penicillin showed no antagonism. The addition of smaller amounts of aureomycin or terramycin (0.1-5 µg/ml), which were mainly bacteriostatic, to a rapidly bactericidal concentration of penicillin (12 µg/ml) resulted in a sharp reduction in the bacterial death rate below that shown with penicillin alone (Fig. 2). This interference increased greatly with a decrease in the amount of aureomycin or terramycin; *e.g.,* 0.1 µg/ml of aureomycin showed more interference than did 1.0 µg/ml. Still smaller amounts of aureomycin (0.01 µg/ml) below its bacteriostatic level no longer interfered with the action of penicillin.

During the first 7 to 12 hours of incubation, mixtures of interfering concentrations of aureomycin or terramycin with penicillin killed *K. pneumoniae* at rates approximating those obtained with the interfering drug alone (Fig. 2). After 24 hours of incubation, however, the organisms exposed to single antibiotics were likely to multiply whereas those exposed to the mixtures were not viable. This ability of combinations of antibiotics to destroy all exposed bacteria ultimately *in vitro* even though the initial death rate is slower than with penicillin alone has also been noted with enterococci(3,6).

There is no evidence that penicillin interferes in any way with the bactericidal action of effective concentrations of either aureomycin or terramycin; *e.g.,* the rate of death of *K. pneumoniae* in the presence of 10 µg/ml of aureomycin was the same whether or not a bacteriostatic concentration of penicillin was added.

Discussion. Apparently aureomycin and terramycin interfere with the action of penicillin only in those concentrations which are at least bacteriostatic and yet not markedly bactericidal by themselves; *i.e.,* inactive concentrations and highly active concentrations do not antagonize the effect of penicillin. With *S. pyogenes* as test organism, 10 µg/ml of aureomycin interfered with 0.6 µg/ml of penicillin; in the case of *K. pneumoniae,* these same concentrations showed no interference. It seems unlikely, therefore, that the antagonism is due to a chemical inactivation of one drug by the other without reference to the sensitivity of the bacteria concerned.

Preliminary experiments indicate that the interference of aureomycin with penicillin occurs not only *in vitro* but also in mice inoculated with either *S. pyogenes* or *K. pneumoniae*(7). Mice treated with both penicillin and aureomycin showed a higher death rate than did those treated with penicillin alone.

It is of interest that not only chloramphenicol but all 3 of the "newer" antibiotics that are widely used clinically, interfere with the bactericidal action of penicillin. While these agents have certain features in common there is no evidence that they resemble one another chemically. To date no evidence has appeared suggesting that the interference of these drugs with penicillin action is due to a direct physical or chemical interaction. Other possible mechanisms for this "antibiotic antagonism" have been previously suggested(4,6). It might be that the 2 interfering drugs compete with one another for "receptors" on the bacterial cell. No evidence has been produced directly favoring this possibility. One drug might alter the developmental characteristics of the microorganism in such a way as to diminish their susceptibility to other antimicrobial agents. All instances of antibiotic antagonism demonstrated thus far in this laboratory have dealt with an interference of bacteriostatic drugs with the bactericidal action of penicillin. Most of the data are compatible with the hypothesis that the presence of a bacteriostatic concentration of chloramphenicol, aureomycin, or terramycin may interfere with the multiplication of bacteria. Since penicillin principally affects dividing microorganisms such an interference with multiplication might result in interference with penicillin action. Future experiments will have to explore this hypothesis.

While striking manifestations of antibiotic antagonism have been produced experimentally in mice it cannot be inferred that the phenomenon may be of importance in the antibiotic therapy of human patients. It should be noted, however, that the drug levels showing antibiotic antagonism *in vitro* and in experimental animals fall in the range commonly obtained in human body fluids and tissues with ordinary therapeutic doses.

Summary. Aureomycin and terramycin interfered *in vitro* with the bactericidal action of penicillin against *S. pyogenes* and *K. pneumoniae*. The early bactericidal rate was slower with mixtures of aureomycin or terramycin with penicillin than with penicillin by itself. Ultimately, however, the mixtures were more likely to destroy all exposed bacteria than were the single antibiotics. Interference was most marked when bacteriostatic concentrations of aureomycin or terramycin were mixed with actively bactericidal concentrations of penicillin.

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A Serologic Study of Chorionic Gonadotrophin. (18263)

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Army Medical Department Research and Graduate School.*

The antigenicity of chorionic gonadotrophin* has been suggested by Leathem(1), Leathem and Rakoff(2) and others. Chase(3) has demonstrated that the serum of rabbits immunized with gonadotrophic hormones reacted with these hormones in a variety of immunological test procedures. CG in urine is readily detected by various biological methods. If this hormone be antigenic it is conceivable that it might be demonstrated serologically as are certain soluble bacterial antigens when present in body fluids or excreta(4-6). In order to investigate this possibility, studies were conducted in which the urine of pregnant and nonpregnant women were tested for CG by both biologic and precipitin tests.

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* Hereafter designated as CG.

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Methods. A total of 17 rabbits, each weighing 2½-3½ kg, were injected with "APL"[†]. This biologic preparation is a combined extract of human placental tissue and pregnancy urine which contains 1000 International Units (I.U.) or CG per ml. The animals were approximately equally distributed with respect to sex. After a serological base line was established, the rabbits were injected intravenously with 10 I.U. of CG for 3 successive days, followed by 20 I.U. on the fourth day. After 3 days rest the dosage was increased to 25, 50, 100 and 200 I.U. on successive days, respectively. Blood was obtained 5-6 days after the last injection. Following this, the animals were injected again on the first 4 days of alternate weeks with dosages increasing up to 2000 I.U. weekly. They were bled 5-6 days following the last injection of each series. The sera from the bleedings were tested by the precipitin method according to the following procedure: the serum dilutions used were undilute, 1-2, 1-4, 1-8, and 1-16; 0.1 ml of the serum was placed in 3 mm precipitin tubes, and overlayed with "APL" in concentrations of 62.5, 125, 250, 500 and 1000 I.U./ml. The tubes were incubated at 37°C for 1 hour and then read as a ring test.

Antisera which were positive in a dilution of 1-4 or greater when tested with 62.5 I.U./ml were used for the detection of CG in urine from pregnant and nonpregnant women. These urines were simultaneously assayed by standard biological pregnancy tests. The pH and specific gravity of each urine was determined prior to testing. In a preliminary study of the effect of the pH of urine upon the precipitin reaction, it was found that at or below pH 4.0 and above pH 7.0 it is quite possible to obtain false or interfering reactions. Consequently, the pH of the urines used in the precipitin test was adjusted, where necessary, to fall into the range of pH 4.0 to 7.0.

In order to remove from immune rabbit sera any factors which reacted with urines from normal nonpregnant women, the following absorption procedure was conducted. Each serum showing detectable antibodies was tested with a group of urines from pregnant and nonpregnant women. Known nonpregnancy urines which reacted with the CG antisera were used in absorption. These urines were sterilized by filtration and added to the serum in the ratio of 0.2 cc of urine to 5 cc of serum. Then the mixture was incubated at 37°C for 1 hour and stored overnight in the refrigerator. The mixture was centrifuged, the supernatant restored to the original volume and again tested with known pregnancy and nonpregnancy urines. This procedure was continued until the serum no longer reacted with the test group of nonpregnancy urines, but continued to react with the known pregnancy urines.

Results. Seven of the 17 immunized rabbits showed detectable antibodies when tested with "APL." Six of the 7 were females. The sera from these rabbits then were used for the detection of CG in urine by the precipitin method. A total of 285 urines submitted for pregnancy tests were studied serologically. The results of the precipitin tests in a group of 85 urines as compared with those obtained with the Salmon(7) rat test[‡] are shown in Table I.

On the basis of the large number of "false

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[‡] These urines were assayed in the Endocrine Laboratory of the Department of Obstetrics and Gynecology, Duke Hospital, under the direction of Mr. Austin A. Salmon.

TABLE I. Comparison of Urines Tested by Salmon Rat Test and Precipitin Tests.

	Salmon test		
Precipitin test*	Positive urines	Negative urines	Total No. of urines
Positive	42	12	
Negative	6	25	
Totals	48	37	85

* Anti-CG rabbit serum + patient's urine.

TABLE II. Precipitin Test of Chorionic Gonadotrophin Rabbit Serum with Urines from 15 Normal Nonpregnant Women.

Sera	No. of urines giving positive reaction	No. of urines giving negative reaction	“APL” antigen control
Crude anti-CG #18,5/25	8	7	Positive
Anti-CG absorbed serum #18,5/25	0	15	”
Normal rabbit serum	0	15	Negative

TABLE III. Comparison of Urines Tested by Frog and Precipitin Tests.

	Frog test		
Precipitin test	Positive urines	Negative urines	Total No. of urines
Positive	103	6	
Negative	6	85	
Totals	109	91	200

positives” in this preliminary study, absorbed serum was considered necessary for use. The serum was absorbed as described in the section under “Methods.” The result of one such absorption is seen in Table II, where it is shown that although the crude rabbit antiserum reacted with 8 of 15 urines from nonpregnant females, the absorbed serum was significantly more specific. Absorbed serum was then used in the serological testing of an additional 200 urine specimens which had been assayed by the frog test(8)§. The results of this comparison are shown in Table III.

It was desired to determine whether the antibodies which gave a positive reaction with “APL” were the same as those which reacted with the urines from pregnant women. Serum was absorbed with “APL” using the method described above. This absorbed serum no longer reacted with “APL” or with the 10 pregnancy urines tested. Similarly, serum absorbed with pregnancy urine failed to react with the 10 pregnancy urines used or with “APL.”

Discussion. The specificity of chorionic gonadotrophin antiserum is of paramount importance if that serum is to be used to detect the presence of the hormone. The first series of 85 urines were tested serologically with unabsorbed antiserum. Positive precipitin tests were obtained with 42 of the 48 urines positive by the rat test; and false positive tests were found with 12 of 37 urines negative by the biologic method. In the next series, 200 urine specimens were tested with absorbed serum and the results were compared with those of the frog test. One hundred and three of the 109 pregnancy urines gave positive precipitin results, while 6 of the 91 urines negative by the frog test reacted serologically, thus constituting 6 false positives. These results indicate that the correlation between the biologic and serologic methods of detecting CG in urine is greater when absorbed antiserum is used. Consequently, before the antiserum is used for test purposes, it is necessary to determine the potency and especially the specificity of the serum. It is not to be implied that the antigen used in preparing this serum was pure. It is conceivable that the antiserum prepared with a more highly purified hormone preparation might give serum of higher potency and greater specificity.

The precipitin test was compared with the Salmon rat test and the frog test. It has been found that the Salmon test as performed at Duke Hospital has an accuracy of 98%. At the present time, it is not possible to state exactly the accuracy of the frog test. For our purposes in this study we have used

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§ Veterinary Division, Army Medical Department Research and Graduate School, and by Dr. Cornelius Burns, Department of Pathology, Georgetown University Medical School.

the biologic test as a basis of comparison; clinical histories can be later used to further assess accuracy.

Included in the series of urines studied were specimens from 3 patients with hydatidiform moles, 1 with chorionepithelioma and 3 with testicular tumors. These specimens were tested either by the rat or frog test, and also by the Aschheim-Zondek method. The serological results with these specimens coincided with the biological assays.

Summary. 1. Seventeen rabbits were immunized with "APL," which is a combined extract of human placental tissue and pregnancy urine. Seven rabbits (6 females) produced detectable antibodies. 2. Two hundred eighty-five urines submitted for pregnancy tests were tested for the presence of CG by biological and serological methods. 3. Native rabbit CG antiserum tends to give many cross-reactions. Therefore, it is necessary to absorb the serum and test it for potency and specificity prior to use in testing urines for the presence of increased amounts of CG. 4. It is suggested that it may be possible to use this serologic method for the detection of pregnancy; the development of an antiserum of somewhat greater specificity would be advantageous.

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Relation of the Adrenal to Glycogen Content and Respiration of Lymphoid Organs. (18264)

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The effects produced by adrenal cortical factors and by epinephrine upon the storage, breakdown and utilization of carbohydrate have been described at length(1-5). Although studies have been made of the influence of the adrenal gland upon the quantities of carbohydrate substances, principally glycogen, in the liver, muscle and certain other organs(6-8), no attention has been paid to the part played by this gland in the carbohydrate metabolism of lymphoid tissue. This is surprising in view of recent work which has shown the importance of the lymphoid organs in a variety of defense reactions within the body and which has stressed the influence of adrenal cortical factors upon this function (9-11).

A knowledge of carbohydrate changes within the lymphoid organs, induced by alterations in adrenal hormone levels, might help to elucidate further the role of this endocrine gland in defense mechanisms. This report describes experiments dealing with the chronic effects of adrenalectomy and cortical extract or epinephrine administration upon the glycogen content of lymphoid organs, the liver glycogen and the respiratory activity of these tissues.

Materials and methods. 1. *Animals.* Female rats of a hardy, closely inbred strain, weighing 140-170 g at the time of operation, were used. The 36 adrenalectomized rats employed were divided into three groups, the first of which consisted of 10 rats, to

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TABLE I. Mean % Glycogen (± Average Deviation).

Wks after operation	No. of rats	Final body wt (g)	Lymph node	Thymus	Spleen	Liver
I(A) . . . SHAM-ADRENX.						
1	2	164	.192 ± .008	.303 ± .065	.150 ± .049	1.900 ± .180
2	2	176	.464 ± .272	.188 ± .102	.128 ± .015	2.043 ± 1.280
3	2	205	.212 ± .140	.110 ± .092	.078 ± .014	2.350 ± .550
4	2	164	.241 ± .082	.170 ± .095	.156 ± .042	1.720 ± .260
I(B) . . . ADRENX.						
1	2	176	.132 ± .029	.116 ± .009	.125 ± .002	2.795 ± .215
2	4	189	.076 ± .038	.036 ± .011	.082 ± .017	.312 ± .213
3	4	181	.099 ± .067	.071 ± .032	.070 ± .012	.489 ± .422
4	2	186	.083 ± .013	.094 ± .013	.048 ± .019	.275 ± .083
I(C) . . . ADRENX. + cortical ext.						
1	2	170	.143 ± .101	.101 ± .069	.177 ± .091	5.530 ± .760
2	4	157	.180 ± .029	.100 ± .022	.134 ± .029	1.799 ± .432
3	2	184	.222 ± .054	.104 ± .037	.106 ± .025	2.760 ± .720
4	2	174	.066 ± .045	.084 ± .022	.034 ± .003	1.395 ± .765
I(D) . . . ADRENX. + epinephrine						
1	4	170	.082 ± .062	.072 ± .043	.097 ± .025	1.018 ± .388
2	3	169	.657 ± .365	.164 ± .110	.108 ± .074	3.857 ± .443
3	4	168	.976 ± .126	1.016 ± .673	.149 ± .049	3.596 ± .443
4	3	158	.088 ± .025	.061 ± .022	.070 ± .020	1.798 ± .371

which was administered adrenal cortical extract* in dosages of 1-2 cc daily, starting on the day of operation. The second group of 14 adrenalectomized rats received epinephrine (adrenalin hydrochloride, Parke, Davis and Co.) in dosages of 200 μg daily, beginning with the day of operation. Half of this group received 1 cc of 1-10,000 epinephrine twice a day, while the remaining animals received 1 cc of 1-5,000 epinephrine once a day. The third group of 12 adrenalectomized rats received no hormone. Eight animals subjected to sham-adrenalectomy received no hormonal treatment. All adrenalectomized animals were maintained on 1% saline given as drinking water.

2. *Glycogen determinations.* The method used was essentially that of Good, Kramer and Somogyi(12) and Shaffer and Somogyi (13). The animals in the 4 groups were killed in the forenoon, at 1, 2, 3 and 4 week intervals after the operation. Determinations were made upon the liver, spleen, thymus and pooled lymph nodes from the iliac, mesenteric and axillary regions. The rats were anesthetized lightly with ether, rapidly exsanguinated by cardiac puncture, and the organs removed. Especial attention was paid to ensuring rapid introduction and dissolution of the tissues in the 30% potassium hydroxide. Wet weights of the organs were then determined. The liver was the first tissue to be excised, and the right lobe was used consistently for the determinations. Except for thin slices which were utilized in the respiration studies, the complete spleens, thymuses and pooled lymph nodes were used for glycogen determinations.

3. *Respiration studies.* The reduction of 2,3,5 triphenyl tetrazolium chloride(14) was utilized to ascertain the respiratory activity of the various tissues. A ratio between the colorimeter reading and the weight in mg of dry tissue, served as an index of relative respiration.

Results. 1. *Glycogen content of liver and lymphoid organs.* Table I A, B, C and D summarize the data for the weekly effects of adrenalectomy and replacement therapy upon the glycogen content of lymphoid tissues and liver. Table IA indicates that the liver and splenic glycogen concentrations in the sham-adrenalectomized rats are fairly constant over the 4-week period; thymic and lymph node glycogen shows some fluctuation from week to week, but it is doubtful that these variations are significant. In the adrenalectomized animals (Table IB), the glycogen levels in the lymphoid organs and liver drop sharply after the first week and remain low during the ensuing 3 weeks. Liver glycogen values in the adrenalectomized animals treated with adrenal cortical extract (Table IC) are significantly above those in the sham-adrenalectomized rats at the first week. Thereafter, they descend to levels approximating those of the controls. In the adrenalectomized group given cortical extract, splenic, lymph node and thymic glycogen values are maintained at control levels until about the 3rd week, after which time they tend to fall abruptly. Adrenalectomized rats treated with epinephrine (Table ID) show a rise in liver, splenic, thymic and lymph node glycogen, maximal levels being attained by the 2nd or 3rd week. For the thymus and lymph nodes, this represents an increase in glycogen values above those in the sham-operated animal. At the 4th week, the glycogen in these organs, with the exception of the liver, is depleted to levels characteristic of the adrenalectomized rat. Fig. 1 illustrates the combined data for the 4-week period. The histograms indicate the depletion of the glycogen levels in the lymphoid tissues and liver following adrenalectomy. The greater effectiveness of epinephrine over the cortical extract, in the dosages employed, is also apparent from this figure.

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* We wish to thank Dr. D. A. McGinty, Parke, Davis and Co., for generous supplies of adrenal cortical extract (1 cc contains 167 μg of Compound E equivalent).

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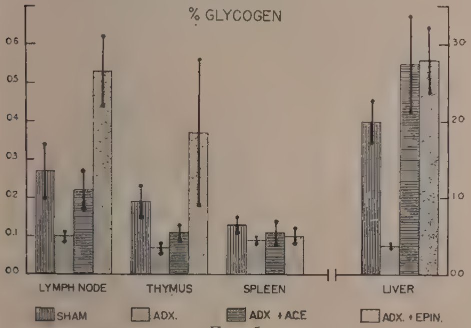

Fig. 1.

Mean % glycogen (combined 4 weekly values) of lymphoid organs and liver as affected by the procedures indicated in graph. Vertical lines represent values of twice the standard error of the mean.

TABLE II. Mean Thymic and Splenic Weights (± Standard Errors).

Treatment	No. of rats	Final body wt (g)	Thymic wt (mg)	Splenic wt (mg)
Sham-Adrenx.	8	177	226 ± 46	623 ± 79
Adrenx.	12	183	346 ± 15	552 ± 111
Adrenx. and Cort. Ext.	8	171	175 ± 23	566 ± 26
Adrenx. and Epineph.	13	166	176 ± 23	495 ± 33

2. *Thymic and splenic weights.* Adrenalectomy results in an increase in thymic weight, which attains a maximum at approximately two weeks following the operation. The weights then fall off, approximating the values in the sham-adrenalectomized rats by the 4th week. Both cortical hormone and epinephrine tend to prevent the thymic hypertrophy, the maximal inhibitory influence being observed at approximately 2 weeks subsequent to adrenalectomy. Thereafter, these hormonal effects are no longer in evidence. Splenic weights were not significantly influenced by adrenalectomy nor by the hormonal treatments employed. The combined data for the 4 weekly periods are presented in Table II.

3. *Respiration studies.* The tissues of the adrenalectomized rats show a greater ability to reduce tetrazolium than those from the sham-operated animals. This effect is seen most markedly during the 1st and 2nd week following adrenal removal. By the 3rd or 4th week, the respiratory activity has fallen to the level seen in the sham-operated rats. Both adrenal cortical extract and epinephrine prevent the rise in respiration noted in the adrenalectomized animal. Here again, the most marked effects are observed during the 1st or 2nd week of treatment. By the 3rd or 4th week, the depressing influence of the hormones upon tetrazolium reduction by the various tissues is no longer seen. Fig. 2 presents the combined data for the 4 weekly periods.

Discussion. As far as the authors are

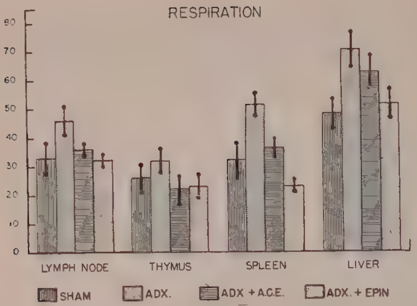

FIG. 2.

Mean respiration—tetrazolium reduction (combined 4 weekly values) of lymphoid organs and liver as affected by the procedures indicated in graph. Vertical lines represent values of twice the standard error of the mean.

aware. these studies are the first to show a relation between the adrenal gland and carbohydrate factors in lymphoid tissues. It is quite apparent that the glycogen content of the combined lymphoid organs is considerable, and that adrenalectomy depletes the glycogen levels of lymphoid tissue as well as that of liver. Both adrenal cortical extract and the medullary hormone restore lymphoid and liver glycogen towards normal in the adrenalectomized rat, this effect being noted particularly at the 2nd or 3rd week of treatment. Although Wagner(15) claims that glycogen is not present in the peripheral lymphocyte, Smith and Thomas(16) have demonstrated its presence in the thymus where it is confined to the small lymphocytes. Thus it is possible that the adrenal hormones affect lymphoid organ glycogen through an action on the contained lymphocytes. The present experiments indicate that the hypertrophy of the lymphoid organs (*e.g.* thymus) following adrenalectomy is accompanied by a decrease in the concentration of glycogen within them, while the partial dissolution of these tissues, caused by the adrenal hormones, occurs concomitantly with an increase in their glycogen concentration. A study is in order, correlating the cytological alterations (*e.g.* ratio of small to large lymphocytes) in the lymphoid organs, to their glycogen content in both the acute and chronic experiment entailing hormone administration.

The tetrazolium studies on tissue slices indicate that there is an increase in the respiration of lymphoid organs and liver after adrenalectomy. These results harmonize with the observation that peripheral oxidation of carbohydrate is accelerated in the adrenalectomized animal(17), but are at variance with the *in vitro* results of Tipton(18,19) who reported a decrease in the respiratory activity of liver tissue following adrenalectomy. The present observations that cortical extract induces a decrease in the respiration of liver and lymphoid tissues, are in accord with the concept that this hormone depresses carbohydrate oxidation within the organism(20, 21) and also with the finding that it inhibits the oxygen consumption of liver slices(22). Correlation of the respiration results to the glycogen levels demonstrates an inverse relation between these factors in the organs of the various groups examined. In this connection, Dempsey and Wislocki(23) have suggested, from their histochemical studies, that glycogen deposition is associated with a deficient circulation, or sluggish metabolism, or both.

The present work points to the possibility that carbohydrate components, as well as protein(10), within the lymphoid organs, may become available to the organism for use in stress, and moreover, that the adrenal hormones which cause release of these factors from the lymphoid tissues, induce, at the same time, a synthesis of glycogen within the remaining and regenerated cells.† Con-

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† Recent evidence indicates that the adrenal cortical hormones induce lymphoid dissolution primarily in the acute experiment (*e.g.* 6-12 hr following a single injection of hormone); with continued injections, lymphoid organ regeneration, and even hyperplasia may occur(11,24).

sidering the extent of the total lymphoid mass to be in the vicinity of 1-3% of the body weight, and the high lability and great regenerative capacity of lymphoid tissue, the turnover during stress of chemical factors contained within these organs may be greater, actually, than that occurring within the liver.

This investigation emphasizes the tendency of epinephrine to mimic, in the adrenalectomized rat, the action of adrenal cortical factors upon the various phenomena examined. The possibility of adrenal cortical rests in the adrenalectomized animals has been considered, but careful examination at autopsy, of the animals injected with epinephrine, only occasionally revealed the presence of small fragments of accessory tissue. It appears more likely that epinephrine, independently of the pituitary-adrenal cortical axis, may initiate, in the chronic type of experiment herein described, reactions which produce some of the end results found in animals treated with cortical factors. It is probable, however, that the mechanisms set into play by the two types of hormones are quite different. The effects of epinephrine upon carbohydrate and respiratory metabolism are complex, and appear to depend upon the dosage and duration of hormone treatment, and whether or not insulin is present(25). The extensive work of Ingle and his associates (5,26,27) has given proof of an extrahepatic function of epinephrine in the eviscerated animal.

Summary. Adrenalectomy in the female rat results in a lowering of the glycogen content of lymphoid tissues as well as liver. Respiration (tetrazolium reduction) of these organs is increased by adrenal removal. Chronic administration of adrenal cortical extract or epinephrine to adrenalectomized rats, restores the glycogen content and reduces the respiratory activity of the thymus, lymph nodes, spleen and liver. Similarly the hypertrophy of the thymus in the adrenalectomized animal is reduced by both hormones. Maximal effects are produced at the 2nd or 3rd week of treatment. By the 4th week, these effects of the hormones are no longer apparent. The demonstrated influence exerted by the adrenal gland upon lymphoid organ glycogen and respiration may be of importance in the general problem of resistance to stress.

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Nitrogenous Excretion in *Colpidium campylum*. (18265)

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As a waste product of protein metabolism, nitrogen is excreted as ammonia, urea, or uric acid. In the ciliate protozoa, the nitrogenous end products have not been identified with any degree of certainty. It has been reported that uric acid is excreted by ciliates (1,2). However, other investigators contend that most ciliates are ureotelic(3,5). There is, moreover, some evidence that ammonia is the principal nitrogenous waste product formed by ciliates. By reason of the fact that bacteria-free cultures were not employed it cannot be concluded whether the reported results stem from protozoan metabolism. A series of experiments was therefore performed in an attempt to ascertain what nitrogenous waste is formed by the ciliate *Colpidium campylum*,‡ which was grown on a carefully controlled, sterile medium.

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Material and methods. Pure, sterile, cultures of *Colpidium campylum* were established in test tubes containing 3% Difco proteose-peptone solution [Elliot(9), rendered fat free (Bloor)(10)]. Each day, for 14 days, 5 test tubes were quantitatively analyzed for ammonia, using the colorimetric method of Folin and Bell(11), and for urea, using a method modified from Folin and Youngburg(12). Qualitative analyses for uric acid (Feigl)(13) were also made. The Accurate population records were kept during the experiment using a dilution method of counting.

Results. The results of the ammonia analyses are summarized in Fig. 1 which shows the mean ammonia production and population respectively plotted against time. A lag phase of growth of 3 days was followed by a logarithmic growth phase. The maximum population of 68,000 colpidia per ml of cul-

uric acid test was sensitive to 1 γ per ml.

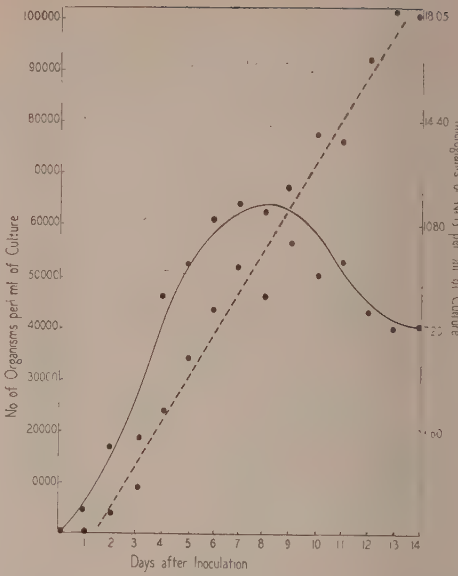

FIG. 1.
Graph comparing 2 curves: ●—— was obtained by plotting number of colpidia per ml against the time in days; curve ● - - -, obtained by plotting the number of γ of ammonia N against the time in days.

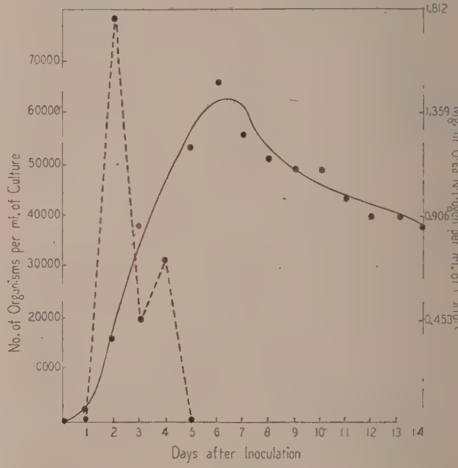

FIG. 2.
Graph comparing 2 curves: ●——, that obtained by plotting number of colpidia per ml of culture against the number of days; ● - - -, that obtained by plotting mg of urea N against the number of days.

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ture was reached on the ninth day after inoculation. Ammonia production increased from the first to the fourteenth day after inoculation at which time 18.0 γ of ammonia nitrogen per ml of culture were produced.

Fig. 2 shows the urea production and mean population plotted against time. The curve obtained is quite similar to that shown in Fig. 1. However, the lag phase is of extremely short duration. Maximum population of 67,000 organisms per ml of culture was reached on the sixth day after inoculation. The population gradually decreased after the sixth day. As is shown in Fig. 2 maximum urea production (1.81 mg of urea N/ml of culture) was reached 48 hours after inoculation. Urea production dropped quickly and was not recorded on the fifth day after inoculation or any day thereafter. No uric acid was detected throughout the 14 days of the experiment.

Discussion. From the results of the present investigations, it might be argued that urea is produced as the principal waste of protein metabolism, and is then hydrolyzed to ammonia. However, if this is true, one would expect a much greater quantity of ammonia than actually obtains. Fig. 2 shows that the amount of urea N present 2 days after inoculation is 1.812 mg per ml of culture. The urea N content drops the following day to 0.454 mg urea N per ml of culture; a decrease of 1.358 mg. Two days after inoculation the ammonia N content is 0.00071 mg per ml of culture. On the following day 0.00143 mg of ammonia N per ml of culture is found. If 1.358 mg of urea N (the decrease in urea N from the second to the third day) were completely hydrolyzed, more than 0.00072 mg of ammonia N per ml of culture would be released. It is therefore, concluded that urea and ammonia are both produced in *Colpidium campylum* and that urea is produced almost solely when metabolic activity is greatest: during the logarithmic phase of growth. Whether urease is present in *Colpidium* is not known. The subsequent fate of urea that is produced is not known at the present time. It must form some nitrogenous compound other than ammonia or uric acid.

Summary. Urea and ammonia were estimated every day for 14 days in sterile cultures of *Colpidium campylum*. Qualitative tests for uric acid were also made. Ammonia production increased linearly with age of culture. Urea was produced only during the logarithmic phase of growth. The cause of the disappearance of urea is unknown. No uric acid was present at any time.

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A Scintillation Counter for Measurement of I^{131} Uptake in the Thyroid Gland.* (18266)

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The uptake of I^{131} by the thyroid is usually measured by a combination of a conventional Geiger counter and scaling circuit (1). In this case, as in most cases of *in vivo* assays of radioisotopes used for therapeutic purposes, the measurements are accomplished by detection of gamma rays, which undergo very little absorption in passage through a small amount of tissue.

The problem inherent in this type of measurement is three-fold: First, the detector cannot be placed in close proximity to the neck, since for accurate estimates the

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1. Symposium on Radioiodine, Brookhaven National Laboratory, July 28-30, 1948.

counter must be able to view the entire gland and detect activity originating in any part thereof. This necessitates counting at a fixed distance from the neck, usually 6 to 12 inches, and results in a deliberate low geometric efficiency. Second, large tracer doses cannot be given in order to obtain higher counter rates because of health hazards. Third, the efficiency of a Geiger counter for detection of gamma rays of I^{131} is low, usually estimated at a value of 0.1% to 1% detection of incident gamma rays from this isotope.

The high efficiency of scintillation phosphors to the detection of gamma rays(2-4) has made their application to this problem an obvious subject of investigation. Much work has already been done on the relative response of various scintillation phosphors (5); but since various phosphors seem to exhibit different relative efficiencies for different types of radiation(6) it seemed advantageous to determine the response of some of the more common commercially available crystals to the gamma radiation of I^{131}. Assuming the decay scheme of Metzger and Deutsch(7) this response would then be that arising from a beam of gamma rays 79% of energy 0.363 Mev, 15% of energy 0.638 Mev, and 6% of energy 0.283 Mev and 0.080 Mev.

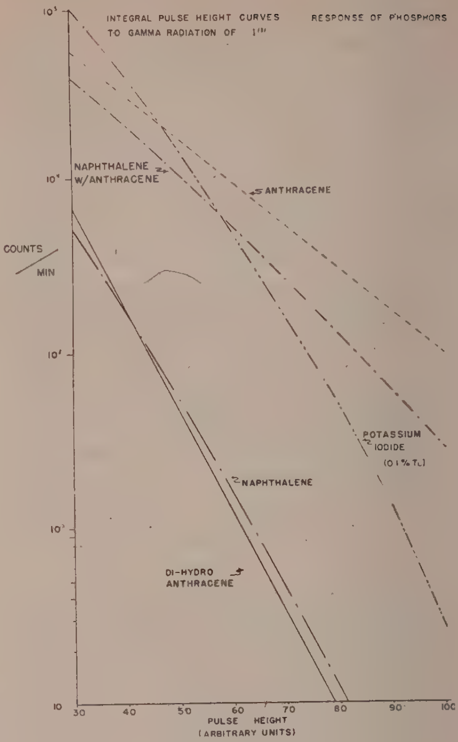

FIG. 1.

In this work 5 phosphors (anthracene, naphthalene, naphthalene with anthracene activator, 0.1% thallium-activated potassium iodide, and dihydro-anthracene)[†] were compared by means of integral bias curves, plotting counting rate versus integral pulse height. The units of pulse height are arbitrary, since they are a function of amplification and not the magnitude of actual pulses received from the electron multiplier tube. The phosphors were mounted on the end of a 5819 electron multiplier tube and exposed to the same source of I^{131} at identical geometric and electronic conditions. Pulses from the tube were fed from a cathode follower preamplifier to a linear amplifier,[‡] discriminator[‡] and scaler. The electron multiplier was operated at room temperature, and at approximately 800 volts. Noise counts from the tube amounted to about one-tenth the total count and were subtracted from each reading.

The comparison curves as shown in Fig. 1 indicate anthracene to have the optimum pulse distribution. The potassium iodide crystal (T1) shows a higher efficiency, as expected, over the organic crystals because of greater density, but a smaller number of

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[†] All obtained from Harshaw Chemical Co., Cleveland.

[‡] Linear Amplifier, Model 204-B, Atomic Instrument Co., Boston, Mass.

large pulses. Recently some 0.5% thallium-activated potassium iodide crystals† have been available and show an efficiency roughly four times higher than those with the smaller amount of thallium. The use of potassium iodide (Tl) crystals would be advantageous where low bias levels could be utilized were it not for the presence of naturally radioactive K^{40}. Assuming a 0.011% incidence of K^{40}, a cubic centimeter crystal of potassium iodide would have approximately 1000 disintegrations per minute, thus making this phosphor of little use for low activity assays. For the remainder of this work, anthracene was used throughout.

Recording equipment. Since the counting equipment was intended for clinical use, the recording circuits were selected with the view towards simplicity, commercial availability, and flexibility. Much of the work with scintillation counters by previous investigators has involved the reduction of tube noise by cooling with dry ice or liquid nitrogen. This was not attempted since the method was considered too unwieldy for clinical procedure. Operation of the tubes at room temperature and adjustment of the bias to eliminate the larger part of the noise such as accomplished by Cassen, Curtis, and Reed(8) was attempted. With the phosphors and tubes chosen, however, the stability and sensitivity desired was not obtained by this procedure.

Instead, the method of using 2 electron multiplier tubes to view the same crystal and reducing background noise by a coincidence circuit was selected. This fulfilled the requirements of simplicity and commercial availability since small amplifiers together with a coincidence circuit, have been recently manufactured.§ These units are small and compact and have given no trouble in operation. The power supply as provided with this unit is unregulated, however, and because of the instability of the power line and the large amount of transient electrical noise pulses, separate power supplies were constructed and employed. The high voltage negative supply was modeled after the circuit of Bair and St. John(9). This embodies a high frequency filter for the transient electrical noise and isolation from all grounds except at the output terminal to inhibit ground currents. The regulation is accomplished by miniature voltage regulator tubes. The low voltage power supply was a conventional regulated power supply based on the supply used for the linear amplifier of Jordan and Bell(10). This combination has performed satisfactorily with the coincidence amplifiers and has presented no problems of stability. The output of the coincidence circuit is fed to a scaling circuit of a negative 0.25 volt input. The detector head was designed to place the scintillation phosphor as close as possible to the 1P21 electron multiplier tubes and to eliminate any air-solid interface which might reflect the scintillations back into the crystal. To do this, the tubes were first placed with their cathodes at right angles and the tube bases in contact with each other. Over this a lucite block was mounted with 2 holes for the tube and a right-angled cut placed within one-eighth inch of the tube. The phosphors were then cut at right angles, polished, and placed in

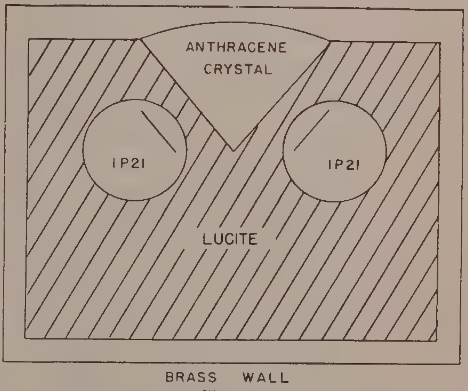

FIG. 2.
Schematic diagram of detector head.

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§ Coincidence amplifier Type 152A W. S. MacDonald Co., Inc., Cambridge, Mass.

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the right-angled groove in contact with the lucite. This is shown in Fig. 2. The air-solid interfaces are then kept at a minimum and can be entirely eliminated for permanent mounting by cementing the crystal and tube face to the lucite with some substance such as Canada balsam. This also keeps the phosphor close geometrically to both cathodes of the 1P21's.

The output from the detector head is fed by a 4 foot cable to the amplifiers and has given satisfactory results operated in this manner. However, a cathode follower preamplifier has been constructed and tests are underway to investigate possible improvements. The preamplifier presents a low capacitance to the anode and thus should give a larger pulse to the amplifier. This is consistent with results since it has been noted that the tubes may be run at voltages of $650 \sim 750$ instead of the usual 850 volts. The investigation of actual improvements in counting efficiency by addition of the preamplifier has not been completed and the following measurements have been taken with direct coupling from the anode of the electron multiplier tube to the amplifier.

Clinical measurements. In the preliminary clinical measurements for uptake of I^{131} in the thyroid gland a triangular anthracene crystal with an outer face of dimensions, 2 inches by ½ inch, was mounted as shown in Fig. 2. In a comparison with a brass walled Eck and Krebs type Geiger counter and a conventional scaling circuit combination as used in the Department of Radiology, University Hospitals, Cleveland, Ohio, an improvement in sensitivity by a factor of 30 to 50 has been obtained. The Geiger counter is shielded by one inch of lead with a one inch diameter hole centered above the gland. As the sensitivity is a function of bias adjustment on the operation of the coincidence circuit the factor of sensitivity improvement is rather arbitrary. The optimum condition of operation has not yet been determined and at the present time the sensitivity selected is mainly determined by the counting rate to which the background is to be restricted.

In the 6-week period that this counter has been in operation, the uptakes of I^{131} in the thyroid glands of 23 patients have been measured and compared with the standard dose of 50 μc used. The average counting rate of the standard was found to be 13,490 c/m with an average uptake rate of 5,908 c/m. In the three months preceding the use of the scintillation counter, the standard Geiger counter as previously described was used. For 23 patients given a 200 μc dose the average uptake was 403 c/m with an average counting rate of the standard of 710 c/m. Thus the counting rate was increased by a factor $15 \sim 19$ although the dose was decreased by a factor of four.

The ratios of the average uptake to average standard with the 2 different dosages and measurement are not identical. This is due to the fact that the 50 μc dose was given to individuals, many of whom were normals, while the 200 μc dose was given only to the patients who were to receive a therapeutic dose of I^{131}. These were thus expected to show a higher uptake of I^{131} and the average uptake would then be expected to show a higher percentage uptake than that of all patients.

Although for routine clinical measurements the counting rates of the standard and the thyroid at one fixed distance are taken, some measurements were taken on the same patient at various distances.

A typical measurement at various distances is shown in Table I together with a comparison of the same standard counted by both Geiger counter and scintillation counter.

TABLE I.

Distance (in.)	Standard		Uptake in thyroid gland,* c/m	% uptake in thyroid*
	Scintillation counter, c/m	Geiger counter, c/m		
16.5	4208	174	1644	39.1
13.5	6492	249	2466	38.1
10.5	10166	423	3831	37.8
7.5	20062	737	7902	38.4

Background: 175 c/m for scintillation counter.
35 c/m for Geiger counter.
* Measured with scintillation counter.

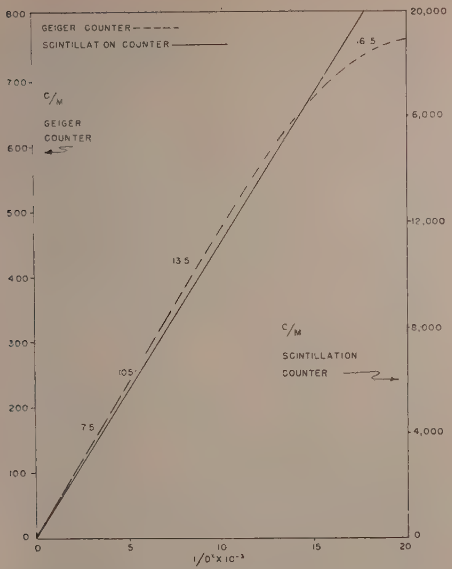

FIG. 3.

Comparison of scintillation and Geiger counter detection at various distances.

A comparison of the two counting rates is shown in Fig. 3 in which the counting rate is plotted versus the inverse square of the distance. At a distance of 7.5 inches, the Geiger counter is seen to deviate from the inverse square law while the scintillation counter is still accurate. The standard of I^{131} was measured in the above case in 50 cc of water to approximate roughly the conditions of actual measurement.‖

Summary. 1. The problems inherent to the measurement of I^{131} uptake in the thyroid gland have been reviewed and the applicability of the scintillation counter for this use discussed. 2. The selection of scintillation phosphor and construction of the counter has been described and compared in operation over a 6-weeks period with a conventional Geiger counter. This improvement of sensitivity has shown to be a factor of 30 ~ 50. 3. The increased sensitivity described has shown to be sufficient to allow an immediate reduction of the tracer dose from 100 μc to 50 μc, with a recent reduction to 25 μc. It may be seen that the dose could be reduced to 10 μc with considerable ease. At the same time a decided improvement over the Geiger counter in counting statistics has been obtained. Based on the previous counting rates the 10 μc standard would exhibit a counting rate ~ 2,700 c/m and an uptake rate ~ 1,200 c/m. Background for such measurements would be 200 ~ 400 c/m.

‖ These clinical measurements were made by John P. Storaasli, M.D. Department of Radiology, University Hospitals, Cleveland, Ohio.

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Effect of Intravenous Injections of Casein Hydrolysate upon Electrocorticogram of the Rabbit.* (18267)

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(Introduced by J. L. O'Leary)

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Enzymatic casein hydrolysate is widely used in the intravenous feeding of protein depleted patients(1). If injected too rapidly side effects of nausea, vomiting and headache ensue. To test the possibility that these symptoms arise from central excitation, a commercial aqueous solution of enzymatic casein hydrolysate† (10% weight/volume, nitrogen content 1.2%) was given intravenously to rabbits while the electrocorticogram was being recorded. The solution was given at a standard rate of 0.3 to 0.5 ml per second and the minimum amount necessary to produce a change in brain activity was determined. Thereafter the amino acids and peptides in the solution were fractionated by adsorption on Amberlite I-R-4, an acid binding resin(2). Both eluates and effluents were tested in rabbits. Finally, samples of the initial solution and its most active fraction were compared by 2-dimensional paper partition chromatography(3).

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* Aided by a grant from U. S. Veterans Administration for research in epilepsy.

1. Elman, Robert, Parenteral Alimentation in Surgery with Special Reference to Proteins and Amino Acids. Paul B. Hoeber, New York, 1947.

† The enzymatic casein hydrolysate was provided by Mead, Johnson and Co. We are indebted to Drs. K. S. Kemmerer and W. M. Cox of that company for extensive assistance in preparation of the concentrate.

The fraction which showed the most marked central effect, as judged by change in brain activity, was obtained as follows: to 1 liter (100 g dry weight) of the enzymatic hydrolysate hydrochloric acid was added to bring the pH to 4.5. The solution was then thoroughly stirred into the 40 g of Amberlite I-R-4 in an evaporating dish and the mixture allowed to stand for 1 hour. The resin was filtered off, washed and eluted with N sodium hydroxide until a pH of 7 was reached; this eluate showed no significant central effect. The resin was eluted again at pH 11.5. The eluate was neutralized with HCl to pH 7 and concentrated to a nitrogen content of approximately 1%. Three separate batches of this maximally active fraction were prepared and a similar effect on cortical activity was produced by each.

Fifty-seven adult rabbits lightly anesthetized with ether were used in the tests. In each animal the cerebral hemispheres were widely exposed for transcortical recording with an ink-writing electroencephalograph. After a 30-minute control electrocorticogram, the test solution was injected at a uniform rate into an ear vein until a change in pattern was obtained, as denoted by the appearance of high voltage paroxysmal discharge, or by assumption of isopotential character (*i.e.*, flattening). In some animals convulsions accompanied the paroxysmal discharge, and death might occur either with or without preceding convulsive activity.

Findings. When the source solution of enzymatic casein hydrolysate was used, changes in the electrocorticogram occurred in 19 of 24 trials with an average injection of 44.3 ml. By contrast the most active fraction, given to 10 animals in 16 trials, required an average injection of only 9.3 ml to produce an effect. Injection of this fraction was followed in 15 trials by marked changes in the recorded brain wave activity in 8 instances with convulsions, and in 4 by death. Effluents were uniformly ineffective, and eluates obtained at other pH's were significantly less active.

Various control solutions were essentially without effect on the electrocorticogram under the standard test conditions. These included: isotonic sodium chloride; unbuffered acid (HCl) and alkaline (NaOH) solutions of pH 3, 5, 7, and 10; 5 and 10% solutions of glucose in water and in physiological saline; solutions of glutathione, glutamic acid, amino acid mixture (10%), acid hydrolyzed fibrin, and acid hydrolyzed casein hydrolysate.

The composition of the enzymatic casein hydrolysate and of the most active fraction (the eluate at pH 11.4) were studied by two dimensional paper-partition chromatography before and after acid hydrolysis.‡ The amino acids were identified by comparison with a reference chromatogram of Dent(3). After the acid hydrolysis of the enzymatic hydrolysate, spots corresponding to glutamine and to a ninhydrin-reactive unknown located below aspartic acid disappeared. On the other hand, the glutamic acid, aspartic acid and valine spots became darker and cystine (cysteic acid), methionine (methionine sulfone) and tyrosine appeared, presumably because these were liberated from peptides which had escaped enzymatic hydrolysis.

The pattern of amino acids found in the pH 11.4 eluate was markedly different from that of the source solution. The quantities of glutamic and aspartic acids were larger and there were several unidentified spots below aspartic acid which gave a strongly positive ninhydrin reaction.

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2. Cannan, R. K., *J. Biol. Chem.*, 1944, v152, 401.
3. Dent, C. E., *Biochem. J.*, 1948, v43, 169.

‡ We are indebted to Dr. E. Roberts of the Department of Anatomy for preparing and interpreting the chromatograms.

The unidentified spots all disappeared following the acid hydrolysis of this fraction. The chromatogram then showed further increases in the concentrations of glutamic and aspartic acids, but other amino acids were also increased. There was no evidence that basic amino acids were present. Their absence is consistent with the fact that Amberlite I-R-4 is an acid-binding resin.

Conclusions. An enzymatic casein hydrolysate was found to contain an agent which produced significant changes in the electrical activity of the rabbit brain when administered under standard test conditions. The agent appeared to be a protein fragment which is adsorbed by Amberlite I-R-4 and yields large amounts of glutamic and aspartic acids when destroyed by acid hydrolysis.

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Influence of Age on Hepatic Response to Choline and Cystine Deficiency in the Rat.* (18268)

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During the past 15 years there has accumulated a considerable body of literature describing the hepatic sequelae of choline and cystine deficiencies. Unfortunately, this literature has been complicated by the fact that the many investigators in this field have employed rats of different strains and ages as well as diets of quite different composition. The present paper describes an attempt to determine the extent to which the initial age of the rat modifies the hepatic response to choline and cystine deficiency in one strain of rats fed one variety of deficient diet.

Experimental. The rats used in these studies were males of the Vanderbilt strain (1). All animals were grown to the desired weight on a commercial feed, housed in individual cages and offered the experimental rations and water *ad libitum.* The basal diet consisted of acid and alcohol washed casein 5, corn starch 54, cottonseed oil 10, cod liver oil 4, shortening 20, salts(4) 4, cholesterol 0.5, cystine 0.6 and choline chloride 0.8. Deficient diets were prepared by simply omitting one of these constituents. In addition, each diet contained the following in mg/kilo of diet mixture: Thiamine 5, riboflavin 8, pyridoxine 5, calcium pantothenate 30, *p*-aminobenzoic acid 75, α-tocopherol 25, biotin 1, pteroylglutamic acid 2, naphthohydroquinone acetate 5, niacin 50. These diets were chosen because of the considerable body of previous experience(2,3) with choline and cystine deficiency in rats fed such rations. With each deficient diet, rats of 3 ages were employed whose initial weights were 50, 150 and 300 g. Since previous experience had indicated that older rats were comparatively resistant to these deficiencies, the experiment was planned in such fashion that, for each of the 3 diets, a representative group of the older rats was sacrificed at about the time the youngest rats on the diet had died. The remaining rats were maintained on the diets and, finally, those which survived were sacrificed after they had been on the diet for 270 days. All livers were fixed in formalin, sectioned and stained with hematoxylin and eosin. On ex-

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* This study was supported in part by grants from the Nutrition Foundation and the Duke University Research Council. Our thanks are due also to Merck and Co. for the crystalline vitamins used and to Miss Ludie Avery for technical assistance.

1. Wolfe, J. M., Bryan, W. R., and Wright, A. W., *Am. J. Cancer,* 1938, v34, 352.

2. Handler, P., and Follis, R. H., Jr., *J. Nutrition,* 1948, v35, 669.

3. Handler, P., and Dubin, I. N., *J. Nutrition,* 1946, v31, 141.

4. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition,* 1937, v14, 273.

TABLE I. Incidence of Hepatic Damage in Choline and Cystine Deficiency.

					Liver		
Initial wt, g	Sacrificed, No.	Died, No.	Time on diet, days	wt change, g	Necrosis, No.	Fibrosis, No.	Fatty, No.
Choline deficient							
50		6	112	+ 8	2	4	4
50	3		270	+ 61	0	3	3
150	5		181	+ 12	0	2	3
150		3	209	— 49	0	2	3
150	6		240	— 24	0	3	6
300		9	165	—118	3	2	6
300	10		273	— 78	0	4	9
Cystine deficient							
50		10	51	— 20	5	0	0
150	6		56	— 57	0	0	0
150		9	91	— 80	3	0	0
300	6		75	—115	0	0	0
300	1	12	143	—184	5	0	0
Choline and cystine deficient							
50		10	56	— 22	0	0	0
150	6		74	— 76	0	0	3
150		8	125	— 88	5	1	2
300	6		75	—127	0	0	3
300		16	170	—180	7	6	5

amination each liver was studied with respect to the incidence and extent of fat accumulation, necrosis and fibrosis. These were graded 0-4+ as described previously(2). The results are summarized in Table I.

Choline Deficiency. In agreement with our previous experience, the response to choline deficiency proved to be quite variable. Of the twelve 50 g rats, 9 died during the experiment but at times varying from 44 to 195 days. Unfortunately 3 of these livers were badly autolyzed and could not be satisfactorily examined. The remaining 6 of the 9 livers in this series showed 1-3+ fibrosis. Two rats showed minimal necrosis and all of those which had gained weight over the experimental period had marked fatty livers as did some, but not all, of those which had lost weight. There was a definite correlation between the length of life and the extent of scarring and fat deposition. Five of the 150 g rats were sacrificed after 18 days. Of these, 3 had extensively fatty livers but showed no necrosis and only 2 evidenced a minimal fibrosis. Of the remaining rats in this group, 66% survived the entire experimental period but all showed fatty livers and half were fibrotic. Like the 50 g rats most of this group gained weight for all but the last month of the experiment and then lost rapidly.

None of the 300 g rats were sacrificed at a time which now permits comparison with younger rats. All of this group lost weight throughout the experiment, yet half of them were alive at 9 months and 15 of the total of 19 had fatty livers. Yet, only 6 showed any fibrosis. Of the total of 28 rats of the two older series which either died of themselves or were sacrificed at the end of the series, only 7 showed a 3+ fibrosis while 17 showed no fibrosis at all despite the fact that 24 of these rats had fatty livers. These findings are in agreement with the impressions gained previously in studies not designed to this end, that hepatic damage due to choline deficiency, beyond the stage of fat accumulation, is much more readily obtained in young than in adolescent or mature rats. The severity of the fibrosis in younger rats was almost entirely dependent upon their survival time but this was not true in the older rats. It is of considerable interest that many of the initially larger animals probably lived for 9 months with massively fatty livers which did not progress beyond this stage. Only under special conditions(3) has this been observed consistently in younger rats.

Cystine Deficiency. In general the relationship observed in choline deficiency also obtained in cystine deficiency, *viz.*, younger rats were much more susceptible than older rats in that they succumbed much sooner. All the rats of the entire series lost between 1/2 and 2/3 of their initial body weight. The mean survival time of the 10 50 g rats was 51 days while, of the older groups allowed to run their course, the survival time of the nine 150 g rats was 91 days and of the twelve 300 g rats, 143 days. Only one rat of the entire series, whose initial weight was 300 g, survived the entire experimental period. In 5 of the 50 g rats, 3 of the 150 g rats and 5 of the 300 g rats were found characteristic necrotic livers. It is noteworthy that, of the 150 g group, the 3 necrotic livers were found in the 3 rats which survived longest.

None of the 13 animals in the 2 older groups, sacrificed at 56 and 75 days respectively, showed any histological abnormalities in their livers. The relatively low incidence of hepatic necrosis among the cystine-deficient animals, despite their uniform survival times and invariable death, raised the problem of the cause of death in the animals with essentially normal livers. A control experiment was run in which 10 rats of each of 3 age groups were fed the basal diet supplemented with both choline and cystine and maintained on this diet for 175 days. At this time, 2 of the 50 g rats and only 1 of each of the other groups had died. Thus the mortality rate on the cystine-supplemented ration was much lower than that in the cystine-deficient series and it must be concluded that early death in the latter group must be ascribed to cystine deficiency even when their livers appear to be essentially normal.

In an attempt to determine the cause of death in those cystine-deficient animals showing no hepatic lesions, another 12 rats weighing 50 g were fed this diet until they died. Sections were made of heart, lung, liver, spleen, pancreas, kidney, adrenal, testis, trachea, thyroid, parathyroid, esophagus, stomach, intestine, eye, brain, skin, bone and marrow. Other than the hepatic necrosis found in half the animals, no lesions were found in any organ. Except for a moderately atrophic skin all tissues were entirely normal in appearance.

Choline and Cystine Deficiency. As in uncomplicated cystine deficiency, all the animals on this diet died during the experimental period. Again, the younger rats were considerably more susceptible than were the older rats, the mean survival times being 56, 125 and 170 days for the 3 age groups, respectively. It is noteworthy that rats deficient in both choline and cystine lived significantly longer than did rats deficient in cystine alone. All rats lost weight throughout the experimental period. The livers of the young rats that died of this dual deficiency were entirely normal in appearance. No explanation is available to account for the protective influence of choline deficiency against the hepatic necrosis of cystine deficiency but it appears likely that the cause of death was similar to that in uncomplicated cystine deficiency. The lack of fatty livers due to the choline deficiency is entirely in accord with all previous experience with choline deficient young rats which lose weight markedly during the experiment. Half of the 150 g rats sacrificed at 74 days had appreciably fatty livers but were neither fibrotic nor necrotic. However, when allowed to progress, half of the remainder developed necrotic livers, presumably due to the cystine deficiency, while only one liver in this group was at all fibrotic. Of the 300 g rats, again half of those sacrificed at 75 days were found to have fatty livers but even these were neither fibrotic nor necrotic. Of the 16 animals allowed to progress, 7 were found to have markedly necrotic livers while 6 were fibrotic. Since only 2 of the livers were both fibrotic and necrotic, it appears likely that these were of different origin, the necrosis having been due to cystine deficiency. The lack of fibrosis in the 150 and 50 g series was probably due to the fact that they did not survive for a sufficient length of time nor had they sufficiently fatty livers. It is of interest that the incidence of fibrosis in the 300 g rats deficient in choline and cystine was of the same order as that in the equivalent choline deficient, cystine fed animals. Most, but not all, of those livers were also fatty, in keeping with the concept that the fibrosis is contingent upon a preliminary fatty period(3). However, it is difficult to understand the maintenance of fatty livers in rats which lost 1/2 to 2/3 of their body weight during the experimental period.

Summary. A study has been made of the variation with age of the response of rats to low casein, cholesterol supplemented diets deficient in cystine and/or choline. Young rats succumb much more readily than do older rats to the effects of dietary deficiencies in cystine or choline. While choline deficiency almost invariably results in hepatic fibrosis in young rats, in older rats it frequently causes nothing but fatty livers even after 8 months. The incidence of hepatic

necrosis due to cystine deficiency in young rats was about the same as that observed in older rats but the young rats developed such lesions in 1/3 the time required in older rats. Many rats of all ages have been observed to die of cystine deficiency with no detectable histological abnormalities in any of 19 organs examined. Young rats, fed a diet deficient in both choline and cystine live somewhat longer than simply cystine deficient animals but show no hepatic damage. Older rats on such rations develop livers which are necrotic, fibrotic, and fatty despite a loss of as much as two-thirds of their initial body weight during the experimental period.

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Blood Levels of I-131 after Tracer Doses in the Diagnosis of Hyperthyroidism. (18269)

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Up to the present time, all methods employing radioactive iodine in the diagnosis of thyroid function have depended upon the well established fact that the hyperactive thyroid gland selectively concentrates and fixes larger amounts of I-131 than does the normal organ. This phenomenon has been measured in 2 ways; first by direct counting over the gland with a suitably arranged Geiger-Müller tube and second, by measuring the excretion in the urine during a fixed period after the administration of the tagged iodine. Both of these methods leave much to be desired so far as results consistent with the clinical state of the patient are concerned. There is a definite, often considerable, area of overlap between the normals and the hyperthyroids. In an attempt to circumvent these difficulties some observers have attempted to study the blood levels of I-131 after tracer doses and have sought additional information by fractionating the circulating I-131 into its inorganic and protein-bound fractions. These studies have again showed a considerable overlap between the normals and the hyperthyroids and have not given diagnostic information as clear-cut as might be desired. It occurred to us that a new approach might be fruitful. If one attempted to measure *secretion* of the thyroid hormone from the gland into the circulation rather than *fixation* of iodine by the gland more conclusive results might be attained. This has a physiological basis because it is the increased discharge of hormone from the thyroid gland rather than the increased avidity for iodine which accounts for the clinical phenomena of disturbed thyroid function.

Studies reported to date have usually been extended over a period of only 24 hours because it was known that fixation of iodine in the gland is quite complete in that period of time. If, however, one carries the observations over a longer period of time and studies blood levels of I-131, the changes due to fixation in the gland become less significant and changes due to hormone *secretion* dominate the findings. Although there is a difference between the amount of I-131 fixed in the glands of euthyroid and hyperthyroid subjects, these differences are not marked enough for a clear differentiation of the 2 groups of patients. However, at the end of 48, 72 or 96 hours after the administration of the tracer dose of I-131 there is a very marked difference in the level of I-131 in the blood and this difference has diagnostic value. The determination of the radioactivity of 1 cc of blood beyond 24 hours after safe tracer doses of I-131 could not be performed until counters of high sensitivity, such as the flow counters, became available.

Selection of clinical material. A consecutive series of 24 normal and 30 hyperthyroid subjects was selected from the wards of The Mount Sinai Hospital. The normal patients were males and females on the wards of the Medical Services. They were selected because they presented no clinical or laboratory evidences of thyroid disease and their basal metabolic rates were within normal limits in all instances. The hyperthyroid patients all were undoubted clinical examples of this disease. The classical symptomatology of goitre, tachycardia, tremor and increased basal metabolic rate was present in every case. Classic eye signs were frequently present. In many instances the clinical diagnosis was confirmed by the presence of an elevated protein-bound plasma iodine level. At least 3 experienced clinicians agreed on the classification and no equivocal cases were included in this study. In the hyperthyroid patients the basal metabolic rates varied between + 20 and + 80%. In order to determine the effect of impaired renal function on the observations to be reported, a group of euthyroid patients with definite renal insufficiency was also studied. The basal metabolic rate of these patients was normal.

Methods. Carrier-free I-131* was administered intramuscularly in doses of 100 microcuries. Preliminary observations showed that, although the oral route could be used, less consistent results were obtained by this method. Intravenous administration had no advantage over the technically simpler intramuscular route. Blood was drawn by venupuncture at regular intervals. A small amount (1 mg) of potassium iodide as carrier and a drop of 10% sodium hydroxide were added to each blood sample. For the counting of whole blood or plasma, 1 cc was pipetted into a suitable planchette, dried and counted in a Q-gas counter (Model D 46, Nuclear Instrument and Chemical Corporation, Chicago). Protein-bound I-131 was determined by precipitating 1 cc plasma with trichloracetic acid, centrifuging and washing the precipitate 3 times with 15 cc portions of the acid. The final washed precipitate was dissolved in alkali and an aliquot representing 0.5 cc of the original plasma was pipetted into a planchette, dried and counted as before. Control studies showed that this procedure quantitatively removes larger amounts of inorganic I-131 than could be encountered in this study. Corrections were made for resolving time, background and decay to the beginning of the experiment. A dried sample of an aqueous solution of radioactive sodium iodide gave an average of 32,000 counts per second per microcurie with the equipment used.

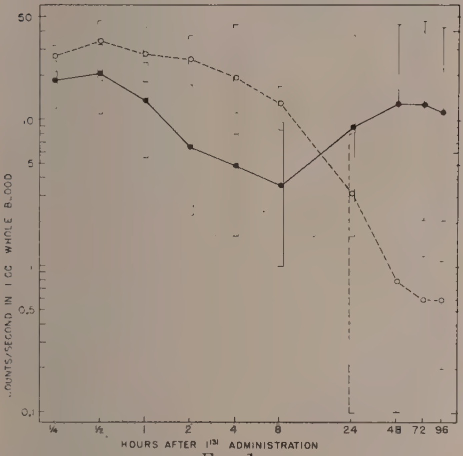

Fig. 1.

Activity in whole blood after 100 microcuries I-131 without carrier; administered intramuscularly. Dots and solid line: hyperthyroids (30 cases). Circles and dotted line: euthyroids (24 cases). Counts are net counts above background and calculated for decay to time of administration. The range of observed values is indicated for each time period. Ten counts per second is equivalent to .001 microcuries I-131.

TABLE I. Counts per Second Due to Protein-bound I-131 per cc of Plasma in Normals and Hyperthyroids After 100 Microcuries I-131 Intramuscularly.

Hr after inj.	4	24	48	72	96
			Euthyroids		
Avg	1.6	1.2	1.4	1.6	1.4
Range	0.6-3	1.0-2	0.4-1.8	1.0-2	0.8-2.2
			Hyperthyroids		
Avg	3.0	14.4	22.4	20.6	22.0
Range	1.0-4	2.6-73	5.6-83.4	5.4-73.6	6.0-77.8

Results. The results of these observations are presented in one figure and 2 tables.

In Fig. 1 where whole blood was counted it will be seen that at 48 hours and beyond there is a sharp separation of the euthyroid and hyperthyroid patients, in fact, there is

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* The I-131 was supplied on allocation by the U. S. Atomic Energy Commission.

TABLE II. Counts per Second per cc of (a) Whole Blood, (b) Plasma, and (c) Protein-bound Plasma I-131, After 100 Microcuries I-131 Intramuscularly in Euthyroid Patients with Impaired Renal Function. Seven cases.

Hr after inj.	4	24	48	72	96
(a) Whole blood					
Avg	26.8	17.3	14.0	7.4	5.0
Range	12.6-44.9	5.1-37.0	3.8-27.2	2.9-20.2	0.2-2.6
(b) Plasma					
Avg	53.0	32.4	27.2	13.1	8.7
Range	23.1-81.2	13.2-71.7	6.4-48.4	6.1-31.9	0.6-26.3
(c) Plasma protein-bound					
Avg	1.4	1.4	1.6	0.8	1.6
Range	1.0-1.8	1.0-1.8	1.0-2.2	0.6-1.4	0.8-2.0

no overlap between the *highest* counts in the euthyroids and the *lowest* counts in the hyperthyroids. It is, of course, not unlikely that some overlap will be encountered as the number of cases studied is increased.

In Table I data on the same patients are presented showing the activity of the plasma due to protein-bound I-131. From these values it is apparent that the high counts found in the whole blood of the hyperthyroid patients at 48 hours and beyond is due to the presence in the plasma of relatively large amounts of protein-bound radioactive iodine; that is, iodine which has been incorporated by the thyroid gland into a protein complex and returned to the circulation as the hormone. A study of these values shows that the thyroid gland in hyperthyroidism produces hormone at a rate some ten times that of the normal gland if we assume that destruction of the hormone proceeds at a constant rate. It is generally accepted that the red blood cells are free of protein-bound iodine.

Table II shows the values obtained in a series of seven euthyroid patients who had renal insufficiency. In these, as in all other cases, the level of activity of the blood at any moment is the net result of several mechanisms at work simultaneously. These are:

1. The transport of the absorbed inorganic I-131
2. The removal of inorganic I-131 by the thyroid from the circulation
3. The excretion of inorganic I-131 by the kidney
4. The return to the circulation of protein-bound I-131 by the thyroid.

Factors 2 and 3 would tend to decrease the radioactivity of the blood while factor 4 would increase it. Factor 1 can be assumed to be relatively constant. It is a well accepted fact that with the impaired renal function the ability of the kidney to excrete iodides is decreased. Therefore, the total radioactivity of the blood should be increased under these circumstances if all the other factors remained constant. The fact that there is no increase in protein-bound I-131 shows that the high values obtained in euthyroid patients with renal disease is due entirely to the continued presence in the circulation of abnormal amounts of inorganic I-131 which the diseased kidney has been unable to clear—a situation quite different from that which exists in hyperthyroidism where the increased radioactivity of the blood is due to the presence of large amounts of protein-bound I-131 which have been returned to the circulation by the overactive thyroid gland. It can, therefore, be stated that a low count at 48 hours or beyond is diagnostic of the euthyroid state. A high count may be the result of thyroid hyperfunction or of delayed excretion due to renal (or "prerenal") disease. In the former the activity is due to protein-bound I-131 and in the latter to inorganic I-131. This distinction can be made by the methods described.

Summary. By prolonging the study of blood levels after tracer doses of I-131 to 96 hours we have been able to measure secretion of thyroid hormone into the circulation rather than concentration and fixation by the thyroid gland. This appears to be the best method presently available for clinical use in the diagnosis of hyperthyroidism. A single blood or plasma sample of one cubic centimeter gives diagnostic values when counted after air drying. The determination of the protein-bound fraction only very rarely gives additional information but can be used to exclude spurious high levels as are occasionally found in cases of renal insufficiency where excretion of the circulating inorganic I-131 is delayed.

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A New Method for the Production of Experimental Bacterial Endocarditis. (18270)

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It was shown previously(1) that discontinuous exposure of rats to simulated high altitudes of 25,000 feet may produce fibrinous vegetations and thickening of the heart valves. The vegetations, except for absence of infection, were essentially similar to those found in human bacterial endocarditis. Because of this similarity and because bacterial endocarditis in man is confined chiefly to previously damaged valves, it was considered possible that rats having heart valves damaged by exposure to altitude would be particularly suitable for the study of experimental bacterial endocarditis.

The need for further investigation of endocarditis is indicated by reports(2-5) emphasizing the difficulties of treatment, the frequent recurrences, and the relatively high mortality (about 30%). Bacterial endocarditis has been produced with varying success in animals, chiefly rabbits, by massive intravenous injections of certain strains of *Streptococcus viridans* and other microorganisms(6-8). However, since the therapeutic response of bacterial endocarditis in man often varies widely with the specific strain of the causative organism, it seemed desirable to find a more suitable laboratory animal that could be infected with greater ease and certainly with a wide variety of organisms isolated from human cases of bacterial endocarditis. As will be shown here, rats exposed to altitude seem to meet these qualifications. Moreover, our findings, indicating that resistance to disease may be lowered by exposure to high altitudes, may be of interest also in aviation medicine and to those concerned with the health of populations living at very high altitudes.

Materials and methods. In a series of 100 consecutive cases of subacute bacterial endocarditis, Niven and White(9) isolated *Streptococcus mitis* in 45 patients, *S. sanguis* in 42, *S. bovis* in 12, *S. faecalis* in 5, and various other organisms in 9 patients. In our study 5 such strains of streptococci were used. Four were isolated from cases of subacute bacterial endocarditis (*S. mitis* JH-26, *S. sanguis* P25 Type 1 and 2, and *S. bovis* P-20*, and *S. mitis* Strain N†) and the fifth was obtained from an infection of the urinary tract (*S.*

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faecalis Strain E‡). Injections of *S. faecalis* were made from 6-hour cultures in beef heart infusion broth containing 0.5% dextrose, while the other bacteria were obtained from 24-hour cultures in the broth without dextrose. Bacterial counts were made on each culture before injection by microscopic examination of a measured volume in wet coverslip preparations. The number of organisms usually ranged from 600 to 800 million per 0.5 ml, the amount injected at one time into a tail vein of each rat. Several groups of Sprague-Dawley rats were exposed 4 hours daily to a simulated altitude of 25,000 feet beginning at 21 days of age following the technique described previously(1). After 3 to 6 months (usually at about 130 days of age), these rats, henceforth designated as altitude rats, received, following their daily exposure to altitude, an intravenous injection of streptococci 4 times weekly for 3 weeks. In each experiment, a group of about 10 altitude rats and 10 non-altitude control rats of similar age and sex distribution was selected and given in random order 0.5 ml of broth from the same culture tube at each injection. Tail blood cultures were generally made 24 hours after the second, sixth, and twelfth injections (tail blood cultures of resistant animals became usually negative in 2 to 4 hours after injecting the bacteria). Autopsies were made immediately after the last blood culture or as soon as possible in case of prior death. The organs were fixed with formaldehyde, and the heart was bisected frontally after fixation. Routine paraffin sections of the tissues were stained with azure eosinate and frequently by Gram's method(10). Each half of the heart was sectioned serially. A few hearts were unsatisfactory for histologic study of the valves.

TABLE I. Mortality and Incidence of Bacterial Endocarditis in (A) Altitude Rats and (B) Non-Altitude Control Rats Inj. with Streptococci.

Micro-organism N	No. of rats	No. died	No. hearts studied*	No. showing endocarditis
S. mitis (JH-26)				
Altitude	10	8	8	8
Control	10	0	9	1
S. mitis (strain N)				
Altitude	6	3	6	3
Control	6	0	6	0
S. sanguis (P-25 type 1 and 2)				
Altitude	21	11	19	11
Control	19	1	15	0
S. bovis (P-20)				
Altitude	11	5	11	4
Control	11	0	9	0
S. faecalis (strain E)				
Altitude	26	19	26	20
Control	20	5	14	7
Total				
Altitude	74	46	70	46
Control	66	6	53	8

* A few hearts are not included because sections of the valves were unsatisfactory for histologic study.

Results. As shown in Table I, 62% of the altitude rats died before completion of the series of 12 injections of streptococci and 66% developed an endocarditis; in contrast, only 9% of the control rats died and only 15% developed an endocarditis. Seven of the 8 control rats with endocarditis received injections of *S. faecalis* and 1 received 12 injections of *S. mitis* JH-26. Thirty of the 46 altitude rats with endocarditis died during the first week; only 4 of the 28 altitude rats surviving 12 injections had endocarditis. There were no significant sex differences in mortality and incidence of endocarditis.

Endocarditis could be predicted antemortem in nearly all altitude rats by the development of a positive blood culture associated with progressive loss in weight, usually ranging from 1 to 5 g per day. The loss in weight was usually greater in males and in the 48 hours preceding death. A few rats with endocarditis lost weight but had negative cultures. Studies in progress indicate that in such cases a second culture taken after a 24-hour rest period may be positive. A positive blood culture with slight loss in weight was

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* Furnished by Dr. J. M. Sherman, Cornell University, Ithaca, N. Y.

† Furnished by Dr. C. H. Binford, U. S. Marine Hospital, Baltimore, Md.

‡ Furnished by Dr. H. Eagle, National Institutes of Health, Bethesda, Md.

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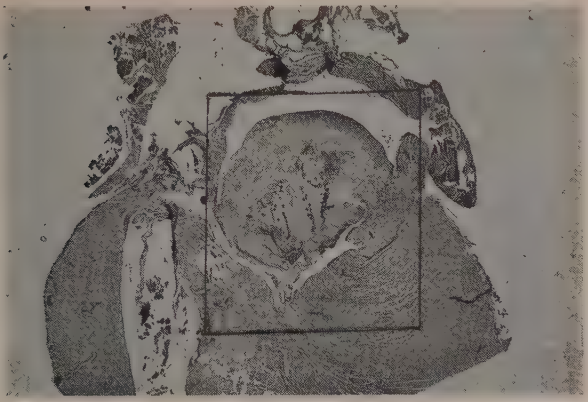

Fig. 1.
Photomicrograph of bacterial vegetation on mitral valve of rat dying after 2 intravenous injections of *Streptococcus mitis* (strain N). Bacterial colonies are dark and lie at several levels in the vegetation. Azure eosinate. Magnification ×5.

seen in some control rats without endocarditis; these animals often showed severe renal changes, an extensive pneumonia, or abscesses in the spleen or other organs.

Rectal temperatures were taken hourly on several occasions, with some animals showing a rise of 1 to 2°C at 2 to 6 hours after an injection. This rise was not considered to be of great diagnostic value because of the wide fluctuations in temperature encountered in many uninjected altitude rats and in some non-altitude rats. A subnormal temperature and great listlessness were noted in some rats for a few hours preceding death. Leucocyte counts taken after a series of daily injections of *S. faecalis* in one experiment ranged between 15,000 and 25,000 in both altitude and nonaltitude rats. Streptococci were found in cultures of the urine aspirated at autopsy from the urinary bladder of a few animals showing focal renal lesions.

Bacterial vegetations were found on about 54% of the mitral valves observed and on 31% of the aortic valves, and in some cases they were present on both valves or on the mural endocardium of the left atrium. No vegetations were found on the tricuspid or pulmonary valves in the animals included in this report. The lesions tended to be most frequent and severe along the line of closure, but often involved other areas and sometimes nearly the entire surface of one or more valve leaflets. Sections of the involved leaflets showed edema, cellular infiltration and marked fibrous thickening with fibroblasts often accompanied by round cells and polymorphonuclear leucocytes extending into the base of the vegetation. The bacterial vegetations were composed chiefly of fibrin often admixed basally with necrotic debris and more superficially with polymorphonuclear leucocytes and red blood cells. Large discrete and confluent colonies of streptococci were found at one or more levels in the superficial and mid-portions of the vegetations (Fig. 1). There were usually some colonies along the surface, but occasionally all seemed covered by a thick layer of fibrin, perhaps accounting for the occasionally negative blood cultures reported above. A few isolated bacteria, often admixed with leucocytes and necrotic debris, were sometimes found in other portions of the vegetations and occasionally in the fibrous tissue at the base of the vegetations. Polymorphonuclear leucocytes were found in variable numbers chiefly at the base and in the superficial portion of the vegetations, and only occasionally near the large bacterial colonies (Fig. 2). The leucocytes were often fragmented and seldom contained bacteria.

In another study(1), 28 rats of corre-

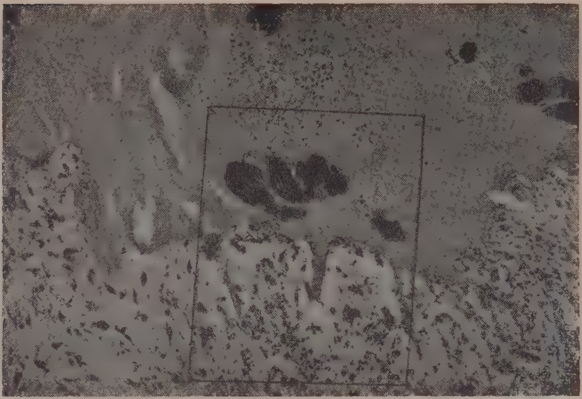

FIG. 2.
Photomicrograph from area at base of vegetation shown in Fig. 1. Azure eosinate. Magnification ×100.

sponding age were exposed to altitude but did not receive streptococci. Nearly all showed thickening and other degenerative changes in the valves, but only 10 (36%) had vegetations (sterile). In the present study, the incidence of bacterial vegetations after injection of streptococci was much higher (66%). This suggests that many bacterial vegetations were formed on damaged valves without antecedent sterile vegetations. It is of interest that several injected altitude animals showed sterile valvular vegetations in areas distant from bacterial vegetations, and 1 animal killed for study following 12 injections of *S. bovis* showed a sterile vegetation on the aortic valve without evidence of infection anywhere.

In many animals there was a severe cellular reaction in the myocardium near the bacterial vegetations, frequently extending to adjoining valves and to the epicardium and adventitia of the large blood vessels. Many of the muscle fibers near the vegetation showed indistinct cross-striations, atrophy, or necrosis; the interstitial tissue showed marked proliferation of fibroblasts and a dense infiltration by small and large mononuclear cells and variable numbers of polymorphonuclear leucocytes and cells with rod-shaped nucleoli as found in *Anitschkow myocytes*. No typical Aschoff bodies were found. The hearts of a few rats showed similar but smaller inflammatory foci in the myocardium distant from the valves. Small hemorrhages were seen occasionally in some of the infected valves and in the epicardium near such valves. The heart of 1 rat dying after 4 injections of *S. mitis* showed a bacterial embolus in a branch of the coronary artery with a perivascular infiltration by polymorphonuclear leucocytes.

The kidneys of many altitude rats, chiefly those that died during the first 4 days after the initial injection, showed hyaline droplet formation, epithelial desquamation or necrosis involving many cortical tubules. These changes were similar to but usually more severe than those seen in some altitude rats not receiving streptococci. A few such kidneys showed erythrocytes or streptococci in the lumens of occasional tubules.

Focal renal lesions were encountered after the fourth day in nearly all animals injected with *S. mitis* JH-26 and *S. faecalis*, but in none injected with the other organisms. There were small areas in the cortex infiltrated chiefly by lymphocytes and showing dilated tubules containing cellular debris, a purulent exudate and sometimes bacteria. In addition there were usually a few necrotic tubules in such areas and rarely a necrotic glomerulus. Similar lesions were seen at times in the medulla, and occasionally a purulent exudate and bacteria were found in the renal pelvis.

One rat that died after 4 injections of *S. faecalis* showed bacterial emboli in many glomerular and intertubular capillaries throughout both kidneys.

Three injected animals had old calcifying sterile renal infarcts (typical of altitude exposure), and 1 had a recent infarct with a markedly congested border densely infiltrated by polymorphonuclear leucocytes. There were many marginal micro-abscesses and scattered clumps of streptococci, some extending into the infarcted area.

The lungs of many rats that died showed intense congestion and often extensive edema, hemorrhages or pneumonia. Several showed centrolobular necrosis in the liver and a few had multiple abscesses chiefly in the lungs or spleen.

Summary and conclusions. Our findings indicate that bacterial endocarditis may be induced readily in rats exposed 4 hours daily to simulated high altitude (25,000 ft.). Twenty-six of 44 altitude rats, but only 1 of 39 non-altitude controls, developed bacterial endocarditis after intravenous injections of *S. mitis*, *S. sanguis* and *S. bovis* obtained from human cases of subacute bacterial endocarditis. Endocarditis was found in 20 of 26 altitude rats and in 7 of 14 non-altitude controls injected with *S. faecalis*. The incidence of endocarditis and the character of the lesions in other organs varied with the species of streptococcus used. It is suggested that altitude rats may be used to study the reaction and to test the effectiveness *in vivo* of various therapeutic measures against specific strains of organisms causing bacterial endocarditis in man. It is also suggested that exposure to altitude may be a useful experimental method for rendering resistant species of animals susceptible to some diseases other than endocarditis.

The authors wish to express appreciation to Dr. Roy Schneiter for valuable suggestions during the course of this study, and to John Simpson for technical aid.

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A Fermentable Non-Reducing Substance in Plasma Following Insulin Administration.* (18271)

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During the course of a study of the action of insulin on glucose oxidation(1) it was found that insulin administration leads to the appearance of a fermentable (*i.e.* liberates CO_2 under the action of yeast) non-reducing substance in the plasma of rabbits. This report concerns the conditions under which this substance is formed.

Experimental. The general plan of the experiments was as follows. After evisceration(2) the rabbits were injected with a priming dose of a potent preparation of uniformly labeled carbon 14 radioactive glucose. They were then maintained for 8 hours with a normal blood sugar level by the constant injection of a radioactive glucose solution calculated to have the same specific activity as that of the circulating plasma glucose resulting from the priming dose. Two groups of eviscerated animals were used and treated similarly except that one group received insulin and the other not. In addition, experiments were carried out on insulinized normal rabbits. The measurement of C^{14} radioactivity, preparation of the carbon 14 labeled glucose, and other experimental details for the eviscerated rabbits not given here has been reported elsewhere(1).

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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The glucose concentration of each plasma sample was determined separately by two independent groups of workers. In the one laboratory the method described by Somogyi (3) was employed. The balance of plasma sample was then frozen in dry ice and sent to the other laboratory. Here the Miller and Van Slyke(4) procedure was used in combination with a micro fermentation using Fleischmann's Baker's Yeast in a manner similar to that described by Reinecke(5). In all cases the values obtained by the two reducing procedures on unfermented plasma checked to within 5 per cent. The non-fermentable reducing substances in each case amounted to 10 per cent or less of the total reducing material.

For the fermentation technic 1 ml of a 20% Fleischmann's Baker's Yeast† suspension (previously washed 5 times with distilled water) is measured in a side arm of a 50 ml flask. In the bottom of the flask is placed 60 mg of pure glucose in 9 ml of water and 1 ml of plasma. The glucose is added in order to obtain sufficient CO_2 for counting purposes. The mixture is made acid to methyl red (pH 3) with a few drops of a saturated solution of citric acid. The flask contents are aerated with nitrogen for 5 minutes. The flask is then closed with a stopper having attached to it on a glass rod a center cup containing 1 ml of 5 N CO_2-free NaOH. After tipping the yeast suspension into the bottom of the flask the sample is incubated with shaking at 30°C for 3 hours. The carbon dioxide is obtained from the center cup by transferring the NaOH with three 1 ml water washes to a 15 ml centrifuge tube. The CO_2 is obtained as $BaCO_3$ by precipitation with 1 equivalent of 2 N NH_4Cl and 2 equivalents of 3 N $BaCl_2$ calculated for the NaOH. The $BaCO_3$ is washed three times with CO_2-free water and once with acetone, dried, and weighed.

Results and discussion. The method of calculating results is illustrated from the data on rabbit 37. The plasma glucose concentration by the Somogyi method was 121 mg % and the Miller-Van Slyke procedure gave values of 119 and 5 mg % before and after yeast fermentation. The value for the glucose concentration is then taken as 114 mg %. The radioactivity of the $BaCO_3$ obtained from fermentation of the mixture of 60 mg of pure glucose and 1 ml of plasma (1 ml plasma = 1.14 mg glucose) at infinite thickness was 544 cts/min/planchet. The calculated specific activity is 83.7. Dilution of the plasma glucose by the carrier glucose is $\frac{61.14}{1.14}$ or 53.6. Thus the specific activity of the plasma glucose is 53.6 x 83.7 or 4500. The specific activity of the injected glucose was measured on the $BaCO_3$ obtained by fermentation.

The data given in Table I for the insulin-treated animals show that the specific activities of the CO_2 obtained by fermentation of the plasma are nearly two times greater than that of the injected glucose. This is in marked contrast to the values obtained without insulin administration. It is obvious that insulin causes the appearance of a non-reducing fermentable substance which contains carbon 14. A comparison of the plasma glucose concentrations determined by reduction in comparison with the values calculated from the fermentable carbon 14 of the plasma show this in an even more striking manner.

Although further work will have to be carried out in order to identify the plasma substance, Van Slyke and Hawkins(6) have

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† The yeast used in these studies was generously supplied by Standard Brands, Inc., San Diego, California.

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TABLE I. Data Showing Appearance of a 'Fermentable Non-reducing Substance' in Plasma of Eviscerated and Normal Rabbits After 8 Hours of Constant Injection of C^{14} Labeled Glucose with Insulin Administration.*

Control						Insulin treated†					
Exp.	Days fast before evisceration	S.A. of CO_2 from plasma fermentation‡	S.A. of CO_2 from fermentation inj. glucose	Plasma glucose det. by reduction§ mg %	Plasma "glucose" calc. from fermentable C^{14} mg %	Exp.	Days fast before evisceration	S.A. of CO_2 from plasma fermentation‡	S.A. of CO_2 from fermentation inj. glucose	Plasma glucose det. by reduction§ mg %	Plasma "glucose" calc. from fermentable C^{14}‖ mg %
14	4	6300	8900	56	40	20	4	5760	3560	62	101
23	3	3540	4200	106	89	22	0	6770	3460	72	138
29	4	2540	2890	108	95	27	1	5015	2240	56	126
30	4	2560	2370	90	94	37	0	4500	3360	114	154
39	0	2500	2890	121	107	40¶	4	2930	2990	112	112
						36	1	2600	1670	150	226
						41	5	2810	1800	73	108
Avg		3488	4250	96	85			4340	2726	91	138

* In all of the experiments the rabbits were eviscerated with the kidneys left intact except experiments 36 and 41, where normal rabbits were used.

† 10 units of Iletin insulin were given I.V. at the start of each hour. We are indebted to Eli Lilly & Co., for generous supplies of insulin.

‡ Specific Activity (S.A.) is the cts./min./mg of carbon.

§ Difference between reducing values before and after fermentation.

‖ An example of this calculation using data for rabbit 37 is as follows: It is assumed that the Specific Activity of the CO_2 obtained from the plasma fermentation is that of the injected glucose and it could not be higher. Actually as can be seen from the values in the control rabbits it is about 20% lower. The S.A. of the fermentation CO_2 is then 3360 (same as the injected glucose) and the dilution of the plasma glucose by the carrier glucose is $x \times 83.7 = 3360$ or $x = 40$. (The S.A. constant of 83.7 is fixed if the counting is correct). The concentration of the plasma glucose under these conditions is 154 mg % $\left(\frac{60 + x = 40}{x}\right)$ instead of the observed value of 114 mg %. Thus the concentration of the unknown compound appears to be present to the extent of the carbon in 40 mg % of glucose.

¶ We are unable to explain the negative results obtained with this animal. Sufficient plasma was not available to repeat the determinations.

pointed out that certain amino acids and α-keto acids have been shown to yield CO_2 under the influence of yeast carboxylase. They attributed the CO_2 from fermentable non-reducing compounds in urine to these sources.

We have made a preliminary survey of possible known substances which could arise from glucose. The following compounds were found not to be fermentable under the conditions reported here: lactic acid, succinic acid, 3-phosphoglyceric acid, glyceraldehyde, glycerol, α-ketoglutarate, dihydroxy acetone, glucose-1-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate. This at first seems very surprising since these phosphorylated compounds are intermediates in the fermentation of glucose and our yeast ferments glucose. This may be due to the fact that intact cells probably do not take up the phosphorylated compounds.

Summary. Data are presented showing that insulin induces the appearance of a 'fermentable non-reducing substance' in substantial quantity in the plasma of eviscerated and normal rabbits after 8 hours of constant injection of C^{14}-labeled glucose.

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Effect of Heat and of pH on Strains of Coxsackie Virus. (18272)

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This report describes studies of the thermostability and resistance to pH of three strains of Coxsackie virus of Group A and two strains of Group B. Results of earlier studies of MM and Lansing poliomyelitis virus are included for comparison.

Procedure. Infected mouse brains or leg muscles that had been stored in a dry-ice box were suspended and clarified by centrifugation. Immediately after preparation, 0.5 ml volumes of the suspensions were sealed in 3 ml lyophile bulbs for immersion in units of 3, in the heating bath. Immediately before and after heating they were chilled in an ice and water bath. For each suspension, one unit was held without heating. The samples were pooled on the day heated and tested in mice on the following day. To determine the time required for the samples to reach the temperature of the bath, a constantan-nickel thermocouple in 0.5 ml of distilled water in a lyophile bulb was used. The lag time was relatively constant about 60 seconds. The pH values of each original suspension and of a heated sample were determined electrometrically. The values lay between 6.3 and 7.4, many between 6.9 and 7.2. Heating of a given suspension did not change the pH. Suspensions were prepared in a 10% solution of beef-infusion broth in physiologic salt solution (broth-salt solution) and in distilled water. They were heated for 30 minutes.

Infectivity was tested by intraperitoneal injection of 0.05-ml amounts into 2- to 6-day-old suckling mice of the Albany standard strain; from 6 to 9 for each dilution tested. They were observed for 2 weeks, but only those affected from the second to the seventh day (for those receiving No. 5096 (Group B), from the second to the tenth day) are included in the results reported.*

MM virus was suspended in distilled water or in physiologic salt solution containing 10% normal horse serum. Infectivity was tested by intracerebral injection of 0.03 ml into 10- to 12-gram mice which were observed for 7 days. Mice dying on the day of injection or the following day were excluded from the results. Lansing virus was suspended in distilled water and tested by intracerebral injection of 0.03 ml into 10- to 12-gram mice which were observed for 21 days. For the tests of pH effect, 10^{-1} mouse-brain suspensions in broth-salt solution were further diluted to 10^{-2} by adding 0.5 to 4.5 ml of solutions of controlled pH. The diluents were Michaelis's veronal-acetate and Sorensen's glycocoll-sodium hydroxide buffers and 0.1 N and 0.01 N hydrochloric acid solutions. They were prepared at approximately the desired pH, and sterilized in a boiling water bath. In each test a 10^{-2} suspension in broth-salt solution was included as a control. The suspensions were held at room temperature under a dark cloth. Tests were made after 1 and 7 days, a fresh container being opened for

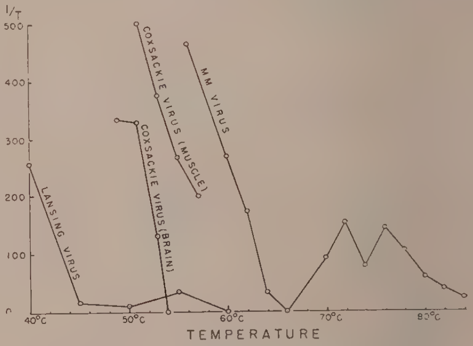

FIG. 1.

Effect of heat on infectivity of Coxsackie virus (Group A, Type 1, No. 48249) as compared with MM and Lansing viruses. 10-1 dilutions were heated for 30 min.

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* Impressions of the activity of virus based on late and early effects were not significantly different from those based on early effects alone. Late paralysis and death were not entirely nonspecific, however. In nearly 900 mice injected with No. 49190 (Group A, Type 2) no effects were observed after the seventh day, whereas roughly 2 mice of every 100 injected with Nos. 48249 and 49191 (Group A, Types 1 and 2) and No. 5096 (Group B), were found paralyzed or dead on the 8th to the 14th day.

each test. The method of testing for infectivity was that described for the heated material. The pH of a sample withdrawn from each suspension on the day of the infectivity test was determined electrometrically. Toxicity of the diluents was ruled out by tests in suckling mice of virus-free samples of each at the extremes of pH used. No growth occurred on blood plates streaked with each test mixture at the time the sample for the pH test was taken.

Results. Effect of heat. The thermosusceptibility of 5 strains of Coxsackie virus was very nearly the same and is in the range of that of poliomyelitis, Y-SK, and mouse encephalomyelitis viruses(1). After 30 minutes at temperatures between 53° and 55°C no infectivity for suckling mice was detected in 10^{-1} brain tissue suspensions in physiologic salt solution containing 10% beef infusion broth. With the exception of 1 strain, No. 49190 (Group A, Type 2), all withstood heating at 49°C with no apparent loss. The viruses were somewhat more susceptible when suspended in distilled water. Muscle-tissue suspensions lost activity at about the same rate per increment of temperature as the brain-tissue suspensions in the range 49° to 54°C, but retained definite activity at 57°C. (Tables I and II.)

MM virus was resistant to higher temperatures, resembling Columbia SK(2) and Mengo(3) viruses. A brain-tissue suspension of MM in distilled water lost a relatively small part of its activity at 56°C. Heating at 62° to 64°C destroyed most of the activity but traces remained even at 86°C. Lansing virus, on the other hand, lost much of its activity at 45°C but retained a trace at 55°C. The apparently greater resistance of muscle than brain suspensions of Coxsackie virus may be due in part to greater concentration. If that is true the results with Lansing virus are not directly comparable. To facilitate comparison the value 1/T(4) has been cal-

TABLE I. Effect of Heat on Coxsackie Virus. Group A. 10^{-1} Brain Tissue Suspension Heated for 30 Minutes.

		No. 48249 (Type 1)					No. 49190 (Type 2)							No. 49191 (Type 3)						
			Dilutions inj.					Dilutions inj.							Dilutions inj.					
Temp., °C	Diluent*	No. of tests	10^{-1}	10^{-2}	10^{-3}	10^{-4}	No. of tests	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	No. of tests	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
N.H.†	B-S	5	38/38	38/38	25/37	3/31	7			45/45	52/54	35/54	5/29	7			39/39	53/54	31/53	12/45
	H_2O	8	61/61	52/59	37/58	9/61	3			23/23	22/23	11/24	1/24	3			24/24	22/23	15/24	5/22
49	B-S	3	23/23	20/22	18/22	2/16	2		15/15	9/15	3/15	0/15		2	8/8	7/7	14/14	14/16	6/8	0/7
	H_2O	2	15/15	15/15	10/16	1/10	1		2/8	0/8	0/8	0/7		1			7/7	7/7	2/8	0/7
51	B-S	4	20/20	22/23	6/22	1/15	2	10/13	3/14	1/4	0/7			2	17/17	15/15	9/16	4/8		
	H_2O	3	14/15	3/16	4/16	0/8	1	4/8	0/8	0/8				1	8/8	3/8	0/8	0/8		
53	B-S	4	16/30	4/32	0/22	0/5	3	10/22	1/23	0/24				4	0/31	0/31	0/32			
	H_2O	3	0/23	0/25	0/14									1	1/8	0/8	0/8			
54	B-S	1	0/8	0/8			2	2/15	1/8	0/14				2	0/16	0/16	1/14			
55	B-S	1	0/8	0/8			2	0/16	0/16	0/8				2	0/15	0/15	0/6			

Denominator = No. of mice inj.; numerator = No. dead, paralyzed, or missing during critical period.

* B-S = Physiologic salt solution containing 10% beef-infusion broth. H_2O = distilled water.

† Not heated.

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TABLE II. Effect of Heat on Coxsackie Virus. Group B. 10^{-1} Suspension Heated for 30 Min.

		No. 5096					No. 49683					
			Virus dil. inj.					Virus dil. inj.				
Temp., °C	Diluent*	No. of tests	10^{-1}	10^{-2}	10^{-3}	10^{-4}	No. of tests	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
N.H.†	B-S	4	23/23	31/31	5/8	2/8	2			16/16	14/16	6/16
	H_2O	1	21/21	23/23								
49	B-S	2	16/16	14/16	7/7		2		16/16	15/15	5/16	2/16
	H_2O	1	5/7	2/8								
51	B-S	1	5/7	1/7			2		8/15	5/16	1/16	
	H_2O	1	3/7	0/7								
53	B-S	2	2/16	0/16			2		0/16	0/16	0/8	
	H_2O	1	1/8	0/8								
54	B-S	1	0/8	0/8								
55	B-S	1	0/8	0/8			1		0/8	0/8		

Denominator = No. of mice injected; numerator = No. dead, paralyzed, or missing during critical period.

* B-S = Physiologic salt solution containing 10% beef-infusion broth.

H_2O = Distilled water.

† Not heated.

TABLE III. Effect of pH on Coxsackie Virus. Group B-Strain No. 5096. 10^{-2} Suspension Exposed at Room Temperature.

		Exposed 1 day					Exposed 7 days				
			Dil. inj.					Dil. inj.			
Diluent	No. of tests	pH	10^{-2}	10^{-3}	10^{-4}	10^{-5}	pH	10^{-2}	10^{-3}	10^{-4}	10^{-5}
HCl	2	1.12-1.16	0/16	0/16			1.14-1.15	1/15	0/15		
	2	2.28-2.29	16/16	16/16	13/16		2.25-2.28	16/16	12/16	2/16	
Veronal acetate	1	2.31	9/9	8/8	3/8		2.28	0/8	0/8	0/8	
	1	4.15	8/8	5/6	3/8		4.14	8/8	6/8	2/8	
Glycocoll-NaOH	2	8.02-8.28	16/16	14/16	12/16		8.10-8.11	15/15	11/16	7/16	
	2	9.31-9.38	16/16	15/15	12/15		9.06-9.15	6/16	4/16	0/8	
	1	9.47	8/8	6/8	4/8		9.70	0/8	1/8	0/8	
Broth-salt	3	7.07-7.25	24/24	16/16	16/24		7.20-7.42	23/23	17/24	7/24	

Denominator = No. of mice injected; numerator = No. dead, paralyzed, or missing during critical period.

culated and plotted against temperature for certain tests. Figure 1 shows the curves for a 10^{-1} suspension of Coxsackie, MM and Lansing viruses.

Results. Effect of pH. Coxsackie virus resembles poliomyelitis virus in its ability to withstand exposure to a wide range of pH(5, 6). All strains survived at room temperature for 1 day in suspensions at pH 2.3 to 9.4, and for 7 days at pH 4.0 to 8.0. No. 49190 (Group A, Type 2) and No. 5096 (Group B) survived for 7 days at pH 2.3 to 9.0. No. 48249 (Group A, Type 1) lost considerable activity at pH 2.3 in 1 day and all activity in 7 days. No. 49191 (Group A, Type 3) was more susceptible than the others at pH 9.4 to 9.6. The virus was more resistant in hydrochloric acid solution than in veronal-acetate buffers of the same pH. Table III presents the results with No. 5096 and is representative of the kind of data obtained.

The author is indebted to Dr. Hildegard Plager, under whose direction the infectivity tests were carried out.

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Staining of Lipids by the Periodic-Acid-Schiff Reaction. (18273)

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Schiff's reagent has afforded a means of visualizing aldehydic groups formed in tissue sections treated with oxidising reagents. In Bauer's procedure for polysaccharides(1) this reagent is used with chromic acid as the oxidant. McManus(2) and Hotchkiss(3) introduced oxidation by periodic acid for the same purpose, while Lillie(4) used successfully K permanganate. It has been widely assumed that the periodic-acid-Schiff staining method (PAS), as applied to tissue sections is specific for saccharidic compounds. Thus the PAS-positive granules in the liver have been described as glycogen granules, the presence of PAS-positive matter in capillary basement membranes and other sites has been considered as a proof that these structures consist of glyco-protein, and the positive staining of myelin sheaths with PAS has been ascribed to the presence of hexose-groups in the molecules of the myelin cerebrosides. Morrison and Hack(5) suggested that the PAS procedure could afford a means of differentiating Gaucher cells, containing the PAS-positive cerebrosides from Niemann-Pick cells. In a recent paper, McManus and Cason(6) adduced further evidence for their view that the PAS technic is specific for carbohydrates. They reported that the positive reaction of tissue structures with PAS can be inhibited by acetylation, but is restored by treatment of the acetylated sections with alkali. Experiments presented below indicate that the PAS method stains unsaturated lipids and sphingolipids as well as carbohydrates.

Experimental. Most experiments were performed using the technic of Coujard as described by Gomori(7). The compounds tested were mixed with plasma, and smeared on marked slides, about 10 compounds per slide. Human and rat livers and kidneys were examined in frozen sections after formalin fixation and in paraffin sections after Zenker's fixation. Specimens of livers of two cases of Niemann-Pick's disease* and spleens of 2 cases of Gaucher's disease were prepared from material which has been for 6 years or more in the laboratory either in the form of unstained paraffin sections, or as pieces of tissue preserved in formalin.

Results. (a) *Pure compounds.* Cerebroside†, sphingomyelin†, a lecithin-cephaline mixture†, saturated and unsaturated fatty substances obtained commercially: tristearin, stearic and palmitic acids; triolein,‡ oleic,‡ undecylenic, ricinoleic, nonenoic, crotonic, maleic, lauric and linoleic acids; and glycogen, failed to form a stained complex after 10 minutes treatment with Schiff's reagent.

After oxidation with periodic acid (according to Lillie's procedure)(1), with K permanganate 1% for 10 minutes, or with 5% chromic anhydride solution according to Bauer's technic, followed by a 10-minutes immersion in Schiff's reagent, the unsaturated lipids stained with the reagent, whereas the saturated lipids remained unstained. The

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* One of them kindly supplied by Dr. Casper of the Beilinson Hospital, Petah Tiqvah.

† Kindly prepared from human brain by Dr. B. Shapira of the Department of Pathological Physiology.

‡ Batches of unsaturated lipids (triolein and oleic acid) which had been stored in the laboratory for a number of months in loosely stoppered bottles stained with the Schiff reagent.

intensity of staining varied, and was most marked in the sphingolipids. Oxidation in hydrogen peroxide of several concentrations gave irregular results, as most lipids came off the slides or turned into grayish substances when treated with this reagent. Positive staining of different intensities after H_2O_2 oxidation was observed with oleic acid, triolein, and the lecithin-cephalin mixture.

When the acetylation technic described by McManus and Cason was applied to smears of pure compounds, the materials were largely dissolved away. Small amounts of unsaturated lipid which remained on the slides after treatment with the pyridine-acetic anhydride reagent gave a positive PAS reaction. Experiments in which the attempt was made to remove the acetyl groups of the acetylated substances on Coujard slides by treatment with KOH 0.1 N according to McManus and Cason were inconclusive, as the smears did not adhere to the glass slides.

(b) *Histological material.* Acetylation abolished the staining ability of the liver cell cytoplasmic granules, and depressed the staining ability of the capillary basement membranes of the kidneys in human and rat paraffin sections. In this respect our findings are not in agreement with those reported by McManus and Cason. Blocks of rat liver and kidney were fixed rapidly in boiling 10% formalin, frozen sectioned and stained immediately afterwards, to avoid as far as possible oxidation of the unsaturated lipids by atmospheric oxygen. The acetylation procedure abolished the staining ability of practically all the liver cell granules, of most of the kidney cell granules, and reduced the intensity of staining of the tubular and capillary basement membranes and of the walls of vessels. After deacetylation of these sections by treatment with potassium hydroxide, the number of granules in the liver cells and kidney tubular cells was greatly reduced. Lipid granules in Niemann-Pick cells and in Gaucher cells stained positively with the PAS procedure. In paraffin sections of the Niemann-Pick cases, most lipid granules were dissolved by the solvents used in the preparation of the sections, but the remaining cytoplasmic substance contained PAS-positive material. The intensity of staining by PAS of the lipid granules in Gaucher and Niemann-Pick cells was reduced by the acetylation. The reduction was most marked in the material preserved in formalin. Treatment of acetylated Niemann-Pick and Gaucher sections with KOH resulted in the restoration of their stainability with PAS.

Discussion. The experiments reported above show that lipids containing double bonds stain with the PAS technic. The PAS reaction appears therefore to resemble in principle the oxidative plasmal reaction described by Verne(8), Gomori(9), Danielli (10) and Cain(11).

It is possible that the oxidation of unsaturated fatty acids by periodic acid follows the general rules of periodic acid oxidation [as formulated by Jackson(12)]. The first step in the oxidation of unsaturated fatty acids by mild oxidizing agents is considered to be the addition of two hydroxyl groups and resulting formation of a dihydroxy acid (Fittig)(13). In this stage, the fatty acid would come to correspond in its structure to the general requirements for periodic acid oxidation, and the molecule would be split with a formation of free aldehydic groups.

The valuable technic proposed by McManus and Cason in proof of the carbohydrate nature of the PAS-positive material of histological sections is seen to be inconclusive in certain circumstances:

1. When the tissue contains a PAS-positive lipid, which is soluble in acetic-anhydride-pyridine, as seems to have occurred in our

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frozen sections of liver cells and renal tubules lining cells.

2. When the tissue contains a PAS-positive lipid which is insoluble in acetic anhydride-pyridine, as appears to be the case in arterial walls and capillary basement membranes.

3. When the tissue contains sphingolipids.

The strongly positive PAS staining of sphingolipids can be attributed to the presence of the amino and hydroxyl groups on adjacent carbon atoms of the sphingosine moiety. In cerebrosides, the hexose group provides an oxidative point, while the double bond of the unsaturated fatty acid serves further to strengthen the reaction. The reduction by acetylation of the staining ability of Niemann-Pick cells preserved in formalin may be atributed to the oxidation of this material during storage. Dihydroxy groups probably appear at the double bond sites as a result of the partial oxidation by atmospheric oxygen, and together with the sphingosine 1 amino 2 hydroxy groups can then be acetylated and thus protected from attack by periodate.§

Conclusions. It must be concluded that the PAS method cannot be used to differentiate Niemann-Pick from Gaucher cells, and that it is not strictly specific for carbohydrates. A positive reaction may be given also by sphingolipids, and possibly by other substances having an amino group and a hydroxyl group on 2 adjacent carbons, as well as by unsaturated lipids.

Summary. 1. Unsaturated lipids stain with Schiff's reagent after oxidation with periodate (PAS reaction). The reaction is not inhibited by previous acetylation according to the technic of McManus and Cason. 2. Sphingolipids stain with the PAS technic whether they contained a carbohydrate moiety or not. Their reactivity may be ascribed to the presence of a hydroxy and an amino group on vicinal carbon atoms of the sphingosine moiety, and to the double bond in the fatty acid radicle. Acetylation reduces but does not abolish the staining ability of sphingolipids, since the double bond is not affected by the procedure. 3. The PAS method is not suitable for the differentiation of Niemann-Pick from Gaucher cells. 4. The ability of liver cell granules and of some renal tubule cell granules in fresh frozen sections to stain with PAS was abolished by acetylation, and largely restored by deacetylation. The fact that the staining of reticulin fibers in walls of vessels was not completely prevented by acetylation is considered as indicative of the presence of unsaturated fatty acid radicles in these structures.

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§ The light staining of the lecithin-cephalin mixture may be ascribed both to the double bond of the fatty radical and to the serine moiety of cephalin.

Arterial Volume Pulses and Peripheral Vascular Responses to Drugs.
(18274)

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The work of Nyboer(1), Bloom, Kaufman, Nims and Nyboer(2), and others has shown that peripheral vasoconstriction produces a decrease and vasodilation an increase in the blood volume pulse of a body segment or organ. Certain difficult problems of instrumentation arise in recording simultaneous volume pulses from a number of total organs.

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Such measurements can readily be made on the arteries which supply the various vascular beds. If these arterial volume pulses reflect changes in the peripheral vascular network beyond them, information on the interrelated responses to physiological stimuli and vasoactive drugs may be obtained.

Method. In our laboratory, simultaneous measurements of this type have been made at a number of points in the circulation. Typical experiments include volume pulses from the renal, splenic, inferior mesenteric and femoral arteries in addition to the pressure pulse from the carotid artery. These have been obtained either by the impedance plethysmograph described by Nyboer(1) or by a photoelectric plethysmograph. In the first case the artery is placed in a lucite trough between two electrodes and uniform contact maintained with a conductile paste. A high frequency signal passed between the electrodes is modulated by the pulsatile electrolyte which represents the blood volume pulse. This is recorded by means of an impedance bridge. The circuit reported by Henny and Boone(3) and Oppenheimer and Chamberlain(4) for the Roentgen Electrokymograph was utilized in the photoelectric measurements. A small mirror is attached to the lower end of a lucite rod which is mounted to face an RCA 931-A photomultiplier. Another lucite rod conducts light from a small flashlight bulb and projects it onto the mirror at an angle suitable for reflection up the bar leading to the phototube. A segment of an isolated artery is interposed in this optical system by placing it on the mirror just below the converging ends of the lucite rods. Both diameter and opacity of the blood vessel modulate the light to yield a pulse tracing similar in form to that obtained with the impedance circuit or with a sensitive pressure plethysmograph. All blood pressure measurements were made from a carotid artery using a Sanborn Electromanometer. A modified Grass eight-channel electroencephalograph was the graphic recorder.

Vasoconstriction and vasodilation. A number of workers (1,2,5-9) have established the fact that the volume pulse of a segment or organ of the body is decreased by peripheral vasoconstriction and increased by vasodilation. Work in this laboratory indicates that the volume pulse in an artery supplying such a segment or organ responds in like manner. We have found that the volume pulse of the total leg as measured by electrical impedance and the volume pulse of the femoral artery measured photoelectrically respond in a comparable manner when cats anesthetized with pentobarbital sodium receive intravenous injections of vasoconstrictor or vasodilator agents.

Fig. 1 illustrates the relationship of mean arterial blood pressure to volume pulse in response to a constricting and dilating agent. It is to be noted that naphazoline hydrochloride (Privine) caused a reduction in femoral volume pulse which was accompanied by a rise in blood pressure, while N-isopropylarterenol (Isuprel) produced a marked increase in pulse height simultaneously with a reduction in mean blood pressure. The exact mechanism by which arterial volume pulses are reduced with peripheral constriction and increased with dilation is not entirely clear. Numerous factors are probably concerned. The volume of blood contained in the artery, as well as variations in the elasticity of the vessel which result from the volume changes, appear to influence the observed pulse height. In the case of the photoelectric pickup, a unit volume of blood added to a vessel already engorged with blood will produce relatively less change in opacity and in cross-sectional area under the photosensitive element than will the same volume of blood added to a less distended artery. Since the capacitative

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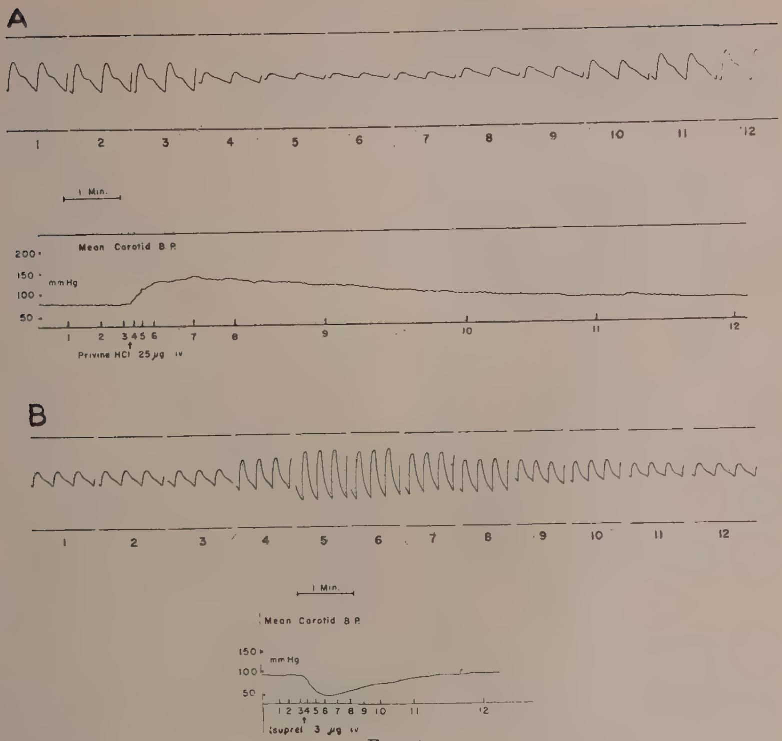

FIG. 1.

Simultaneous recordings of femoral arterial volume pulse and mean carotid blood pressure during circulatory responses to intravenous injection of a constricting (A) and a dilating (B) agent. The numbers under the pressure curves correspond to the samples from the electrical impedance pulse tracing which were taken at a faster paper speed.

coupling employed with the phototube circuit records change from a reference level but not total change, it will show the relative effect of each pulsation but will not indicate the degree of engorgement. To this influence of the geometric factor is added the loss of elasticity which occurs in a distended vessel. Therefore, increasing blood content will have a diminishing effect on the net distention produced by each pulse. Both of these factors probably influence the photoelectric measurements. In the electrical impedance determinations, the geometric relationship is not involved. In this procedure total electrolyte is balanced out by a series resistance between the bridge and the artery and the addition of a unit volume of blood electrolyte causes the same deflection of the galvanometer regardless of the degree of engorgement at the point of measurement. Therefore it is probably the decrease in elasticity of the distended vessel that is largely responsible for the reduction in recorded pulse height.

In a given vascular bed, vasomotor changes may occur as a result of mechanisms which involve the entire arterial system. Active constriction of the arteries and arterioles may of itself oppose the distention of the vessels by an increase in pressure. Conversely a decrease in tone may augment the effect of the pulse.

There is another factor which may contribute to a reduced volume pulse in an artery leading to a constricted peripheral vascular bed: the increased pressure in that vessel may limit the amount of blood entering from the aorta with each ventricular contraction. For example, if the vascular bed supplied by the mesenteric vessels is constricted relatively more than other areas of the body, more resistance to flow is present and less blood (smaller volume pulse) may be expected to enter the mesenteric arteries from the aorta. At a uniform stroke volume, the magnitude of the central pressure pulse in the aorta is controlled by the algebraic sum of the various peripheral factors which oppose blood flow. In the arterial branches we believe these factors are specific for the vascular bed which a given vessel serves. With the extreme peripheral constriction, venous return may be considerably reduced. If this occurs, a reduction in the central pressure pulse width is seen as a result of a smaller stroke volume. This will reduce the volume pulse in the systemic arteries.

An increased volume pulse does not in itself indicate peripheral vasodilation. The effect of the heart must be taken into consideration. If a marked change in stroke volume or a positive inotropic effect does not take place, the peripheral response will occur in a consistent manner. In other words, the arterial volume pulse will increase with dilation and diminish with constriction. With a positive inotropic action such as is produced by epinephrine, an increase in aortic pressure pulse width takes place. This may be attributed to an augmented strength of ventricular contraction and perhaps also an increased venous return. Under these conditions, arterial volume pulses may be increased. Simultaneous records made in this laboratory with a volume pulse recorder and a bubble flow meter show that the increased arterial pulses are associated with an increase in blood flow. Perfusion experiments carried out in the same vascular beds indicate that in certain organs, epinephrine produces vasoconstriction. This apparent discrepancy is clarified if the drug is given intra-arterially or, for the multichannel experiments, into the aorta. Under these conditions, the drug affects the peripheral vessels before it reaches the heart. For somewhat more than one circulation time, the peripheral constriction is apparent and the arterial volume pulses are reduced. Subsequent cardiac stimulation often overshadows a peripheral constrictor action, since arterial volume pulses are increased as an increase in blood flow takes place.

In order to demonstrate the peripheral response in a discrete vascular bed, electrodes were placed on each side of a dog's nose in the region of high vascularity in the nasal passages. An impedance pulse was taken in the manner just described for the isolated artery. Topical application in the nasal passages of amphetamine sulfate or amyl nitrate caused responses of the vascular bed comparable to those noted in the femoral artery after intravenous injection of constricting or dilating agents. This method is routinely used in our laboratory for the evaluation of local vasoconstrictor compounds.

Blood flow. Hamilton and Remington(10) published a study relating cardiac output to the pressure pulse contour in the aorta. This method was elaborated in a further publication by the same authors and their associates (11) and their results substantiated by Huggins, Smith and Sinclair(12). This technic links the pressure pulse with blood flow. Studies in our laboratory show that an arterial volume plus also can indicate relative changes in blood flow. The question of back flow or retrograde flow appears to complicate this mechanism. However, in a peripheral artery, a relatively large backflow is unlikely. The relative volume of the arterial tree increases peripherally. Actual flow measurements made by Gregg and Green(13) on the coronary circulation, where the compression of the vascular bed by the contraction of the myocardium markedly accentuates resistance to flow during the systole, showed only about 10% backflow during the systolic period and none during diastole. Thus it appears that a volume pulse of an artery when related to heart rate can give an indication of blood

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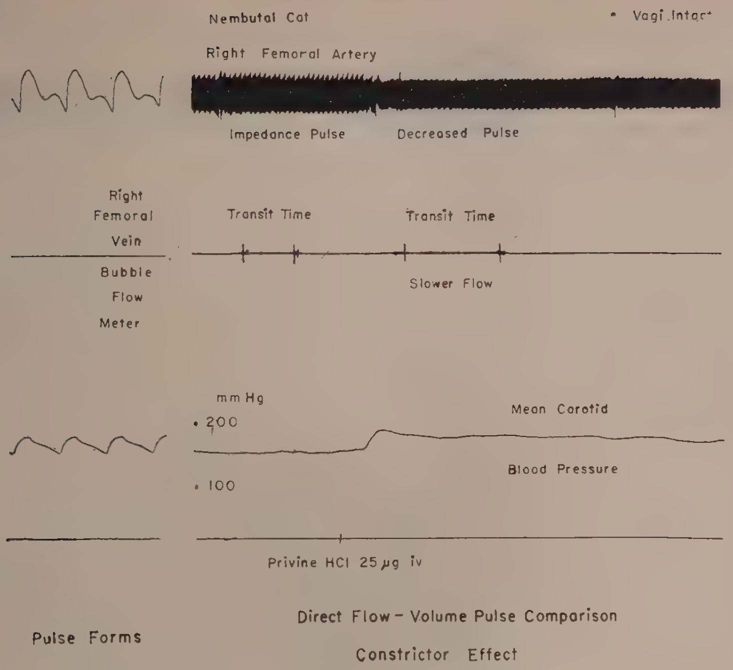

FIG. 2.

Direct Flow—Volume Pulse Comparison, Constrictor Effect. A small segment of record taken at a fast paper speed earlier in the experiment is included on the left to show the form of the impedance volume pulse which is condensed into an envelope by the slower paper speed.

flow through that vessel.

Bloom, Kaufman, Nims and Nyboer(2) published a method for calculating a flow index from a volume pulse tracing. The pulse height is considered proportional to the actual volume pulse. To include the diastolic factor a perpendicular is erected at the beginning of systole and a line drawn to represent the mean diastolic slope. This slope is proportional to the rate at which blood leaves the segment. This line is projected to intersect the perpendicular marking the beginning of systole and an expression of blood flow is obtained. The authors point out that this method does not distinguish between normal and backflow.

To demonstrate how pulse height recorded from an artery varies with peripheral resistance, simultaneous records of volume pulse from the femoral artery and blood flow in the femoral vein were taken along with mean carotid blood pressure. In Fig. 2 the response to an intravenous injection of 25 µg of Privine hydrochloride is illustrated. Here pressure increases while blood flow and pulse height decrease. In Fig. 3 is shown the effect of intravenous injection of 3 µg of Isuprel. The arterial volume pulse again varies inversely with peripheral resistance and reflects the dilation in the vascular bed which the artery serves. These pulses can be recorded from an unopened circulation without the use of an anticoagulant. This is often of considerable advantage in maintaining an experimental preparation in a physiologically responsive state.

Summary. Two methods are described for recording blood volume pulses from body segments and from isolated arteries. Evidence is presented to show that an arterial volume pulse reflects vasomotor change in the vascular bed which that artery supplies peripheral to the point of measurement. Peripheral vasoconstriction and vasodilation can

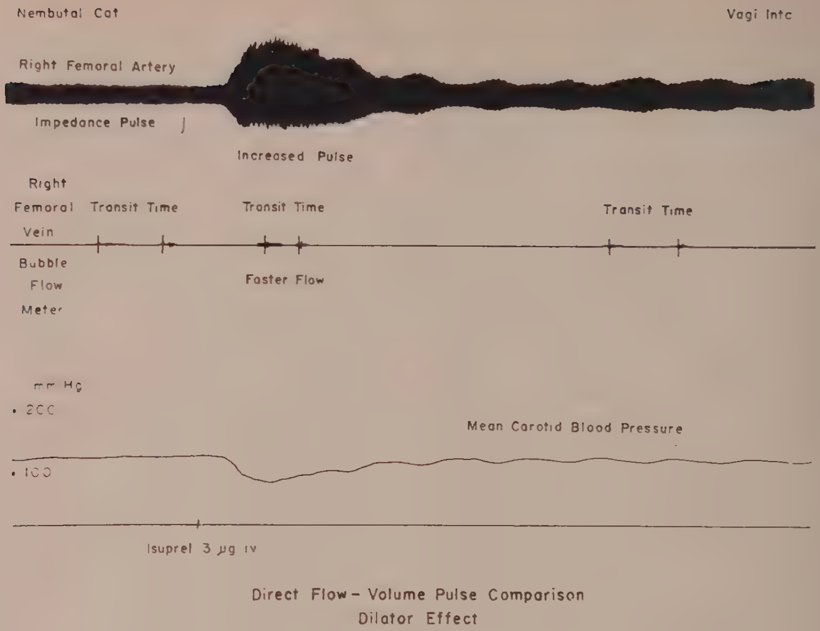

FIG. 3.
Direct Flow—Volume Pulse Comparison, Dilator Effect.

be demonstrated in this manner and indications of relative changes in blood flow can be obtained. With these methods hemodynamic responses to pharmacologic agents can be studied. The initial responses and subsequent adjustments can be followed.

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Influence of Fasting and Adrenalectomy on Normal and Malignant Lymphoid Tissue Composition.* (18275)

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Among considerations of interest in the study of a neoplastic tissue is its responsiveness to specific physiologic stimuli known to have characteristic influences on homologous normal tissue. The well-known involutional response of lymphoid tissue to pituitary-adrenal cortical secretions, and, through their mediation, to a variety of stresses(1), has prompted investigations of the sensitivity of lymphoid tumors to these influences. Such studies have already indicated that in laboratory animals(2,3), and in humans(4), strik-

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ing regression of lymphoid tumors occurs during treatment with pituitary adrenocorticotrophic hormone or adrenal cortical steroids.

Previous studies have shown that the considerable loss in weight and nitrogen content of the pooled lymphoid tissue (spleen, lymph nodes and thymus) which occurs during a 48-hour fast in CBA mice is dependent upon adrenal secretions and fails to occur in the adrenalectomized, fasted animal(5). The availability in mice of the same strain of a rapidly growing transplantable lymphosarcoma which metastasizes only late and consists of cells morphologically similar to normal lymphocytes(6), permitted the extension of such observations to tumor-bearing animals. The present report deals with a study of the weight and composition of normal lymphoid structures and of the tumor as these values were influenced by a 48-hour fast in intact and adrenalectomized mice. Data describing changes in the liver weight and composition of fasted, lymphosarcoma-bearing mice have been presented previously (7).

Methods. Male mice of the CBA strain (Strong) weighing 20 to 25 g and 8 to 9 weeks of age were used. All animals were fed the standard laboratory diet of Rockland chow and, during the period of fasting, were housed in individual metabolism cages with free access to water. Fed mice used as controls for fasting experiments were similarly kept in metabolism cages for the duration of the fasting period, to eliminate the considerable difference in activity which larger cages permit. Mice prepared for fasting experiments were inoculated with a small fragment of lymphosarcoma(6) maintained in the same strain. At varying intervals thereafter, groups of 5 to 7 mice were subjected to a 48-hour fast, while comparable control groups were fed the laboratory diet, as noted above. At the termination of the fasting period, each mouse of both groups was autopsied. Tissues removed routinely for weight and analysis were liver, spleen, tumor, pooled thymus and mesenteric lymph nodes, and a sample of the muscle (gastrocnemius). The entire liver and tumor[‡] were weighed to the nearest 5 mg on a pharmaceutical torsion balance; the spleen, thymus-nodes and muscle sample were weighed to the nearest mg either on a Roller-Smith torsion balance or on an analytical balance in connection with water analysis. Aliquots of each tissue were taken for nitrogen analysis by the micro-Kjeldahl procedure(8). Water content was determined by drying small aliquots of tissue overnight in an oven at 110°C. Tissue protein was approximated by multiplying percent nitrogen by the factor 6.25; tissue lipid was then calculated by difference from the values for percent protein and percent water(9). In some instances (Experiments V, X, XI in Tables I and II), groups of fed mice were autopsied at the beginning of a fasting period to provide initial data with which fasted and fed groups autopsied at the end of a 48-hour fast could be compared. Bilateral adrenalectomy was performed in tumor-bearing mice under ether anesthesia, and the animals maintained continuously thereafter on 1% saline drinking water. Fasting experiments were initiated in 2 such groups of mice 24 hours (Experiment X) and 48 hours (Experiment XI) following operation.

Results. Body weight. As is indicated by the weight data in Table I, fed mice in which the tumor is growing experience a progressive gradual increase in total body weight which is only partly accounted for by the increase in mass of the tumor itself. Since

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						Wt in mg		
Exp.	Group	No. of mice	Age (wk)	Tumor age (days)	Body wt (g)	Tumor	Thymus-nodes	Spleen
					Normal mice			
I	Fed	7	8.1	—	25.3 ± .6	—	103 ± 4	74 ± 2
II	Fed	5	8.0	—	21.6 ± .3	—	92 ± 5	85 ± 2
	Fasted	5	8.0	—	17.6 ± .7	—	68 ± 5	51 ± 4
					Tumor-bearing mice			
III	Fed	5	8.3	2	20.0 ± .9	<10	78 ± 3	74 ± 3
	Fasted	5	8.3	2	15.8 ± .8	<10	53 ± 3	41 ± 3
IV	Fed	5	8.3	6	20.9 ± .9	<10	68 ± 6	127 ± 14
	Fasted	5	8.3	6	15.9 ± .8	<10	39 ± 4	59 ± 6
V	Fed	5	8.3	4	22.4 ± 1.2	<10	70 ± 7	110 ± 11
	Fed	4	8.6	6	22.1 ± .7	279 ± 40	65 ± 6	113 ± 13
	Fasted	5	8.6	6	16.8 ± .7	15 ± 10	50 ± 7	55 ± 3
VI	Fed	6	8.7	7	24.5 ± .5	530 ± 28	78 ± 4	115 ± 9
	Fasted	5	8.7	7	16.4 ± .3	80 ± 31	49 ± 6	55 ± 4
VII	Fed	6	8.3	9	24.0 ± .8	177 ± 30	105 ± 6	77 ± 3
	Fasted	5	8.3	9	19.0 ± .7	117 ± 35	54 ± 6	54 ± 5
VIII	Fed	6	8.1	11	26.2 ± .8	1577 ± 233	80 ± 6	124 ± 7
	Fasted	6	8.1	11	19.2 ± .7	428 ± 112	45 ± 2	65 ± 3
IX	Fed	3	8.4	15	30.2	3580	103†	163†
	Fasted	2	8.4	15	21.1	2340	38	79
					Adrenalectomized, tumor-bearing mice			
X§	Fed	5	8.1	7	20.5 ± .8	<10	81 ± 8	143 ± 20
	Fed	5	8.4	9	21.2 ± .7	244 ± 108	92 ± 3	142 ± 7
	Fasted	7	8.4	9	21.2 ± 1.0	150 ± 60	82 ± 6	137 ± 11
XI‖	Fed	5	9.6	11	22.6 ± 1.0	527 ± 60	95 ± 6	163 ± 9
	Fed	4	9.9	13	22.8 ± .9	1060 ± 221‡	89 ± 6	122 ± 6
	Fasted	6	9.9	13	20.9 ± .5	590 ± 102	80 ± 7	108 ± 11
	Fed¶	4	9.9	13	31.2 ± .7	1980 ± 209	106 ± 16	185 ± 19

* Values shown are means and stand. errors. Stand. errors are not shown for groups of less than 4 individuals.

† Grossly enlarged and thought to be tumor infiltrated.

‡ Group of 7 mice.

§ Adrenalectomized on 6th day following tumor implantation.

‖ Adrenalectomized on 9th day following tumor implantation.

¶ Non-adrenalectomized controls given tumor on same day as remaining mice in Exp. XI.

no significant increase in the water content of muscle (Table II) occurs during the period of tumor growth, the possibility of progressive generalized hydration seems contraindicated. The percent loss of body weight during a 48-hour fast, as calculated by comparing fed and fasted tumor-bearing animals at the termination of the fasting period, similarly increases with the size which the tumor has attained by the beginning of the fast. Fasted mice free of tumor lose, under these conditions, approximately 20% of their body weight, while a similar fast begun when the tumor has been present from 7 to 13 days results in a difference of 27 to 30% between the weights of fasted and fed animals. At least part of this increment of weight loss is accounted for by the inhibition of growth of the tumor itself, as described below. As previously noted(9,10), the adrenalectomized mouse fails to respond to a 48-hour fast with a loss of body weight as great as that found in the intact fasted animal. It is evident from the data of Table I that the presence of a tumor does not alter this inhibition of body weight loss by adrenalectomy. In the case of Experiment X, the surprising failure to demonstrate any mean weight difference between fasted and fed animals was due to

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10. White, A. and Dougherty, T. F., *Endocrinology*, 1947, v41, 230.

TABLE II.* Percent Composition of Water, Protein and Lipid in Tumor, Lymphoid Tissue and Muscle of Fed and Fasted Unoperated and Adrenalectomized Tumor-bearing Mice. Experiment numbers correspond to those in Table I.

			Percent of each component											
			Tumor			Thymus nodes			Spleen			Muscle		
Exp.	Group & No.	Tumor age (days)	Water	Protein	Lipid	Water	Protein	Lipid	Water	Protein	Lipid	Water	Protein	Lipid
	Normal mice													
I	Fed(7)	—	—	—	—	74.9	15.5	9.7 ± .9	78.3	17.6	4.4 ± .4	75.3	20.7	3.9 ± .6
	Tumor-bearing mice													
IV	Fed(5)	6	—	—	—	72.0	12.1	15.9 ± 3.4	79.0	16.6	4.8 ± .3	75.2	19.4	5.3 ± .4
	Fasted(5)	6				79.2	15.3	5.5 ± .8	78.8	16.8	4.4 ± .4	76.1	18.3	5.5 ± .5
VI	Fed(6)	7	73.7	10.2	16.1 ± 1.1	77.3	12.4	9.2 ± 1.1	79.1	16.8	4.1 ± .2	77.7	19.3	2.9 ± .6
	Fasted(5)	7	68.1	11.0	20.9 ± 3.5	80.3	14.2	5.7 ± .5	79.4	17.0	3.6 ± .3	73.1	19.5	7.4 ± 1.1
VII	Fed(6)	9	73.4	12.9	10.3	77.6	14.7	7.9 ± .8	79.1	17.7	3.5 ± .2	74.3	19.7	6.6 ± 1.4
	Fasted(6)	9	73.2	14.3	9.4	80.5	15.0	4.5 ± .4	78.4	18.5	3.1 ± .2	75.4	21.9	1.9 ± .5
VIII	Fed(6)	11	79.3	12.7	7.9 ± 1.8	77.5	14.3	7.8 ± 1.1	79.0	16.4	4.7 ± .2	75.7	19.2	5.5 ± .9
	Fasted(6)	11	80.9	13.7	5.7 ± .4	79.4	15.0	5.5 ± .6	77.1	17.7	5.6 ± 1.2	75.7	21.5	3.2 ± .6
IX	Fed(3)	15	81.5	15.6	2.9	80.5	13.2	6.3	79.8	16.2	4.9	75.9	19.5	2.9
	Fasted(2)	15	81.9	14.9	3.1	79.8	15.1	5.2	78.6	17.4	4.0	77.5	18.9	3.5
	Adrenalectomized, tumor-bearing mice													
X	Fed(5)	7	—	—	—	76.1	14.0	9.9 ± .6	78.9	16.9	4.1 ± .2	76.3	19.8	3.9 ± .5
	Fed(5)	9	74.9	17.2	11.8 ± 4.2	75.4	13.9	10.4 ± 1.9	79.1	16.3	4.6 ± .2	74.5	19.8	5.7 ± .2
	Fasted(7)	9	78.2	14.2	10.1 ± 2.7	78.2	12.6	9.1 ± .4	79.2	15.6	5.2 ± .4	76.5	18.9	4.6 ± .5
XI	Fed(5)	11	75.1	12.7	12.5 ± 3.4	78.4	13.7	7.9 ± .8	79.3	16.2	4.5 ± .2	75.9	19.0	5.1 ± 1.1
	Fed(4)	13	77.5	14.3	8.2	78.7	13.9	7.4 ± 1.3	78.8	16.6	4.6 ± .5	76.8	18.2	5.1 ± .9
	Fasted(6)	13	79.9	14.9	5.4 ± 1.0	78.3	14.9	6.9 ± 1.9	79.1	15.0	5.8 ± .7	76.5	18.8	4.8 ± .6

* Values shown are means and standard errors. Standard errors have been calculated only for the lipid percent, except where groups contain less than 4 mice.

the presence, in several of the fasted mice, of distinct ascites unassociated with any intraperitoneal extension of the tumor.

Tumor weight and composition. The most striking feature of the tumor data is the consistent observation that the mean tumor weight in fasted mice was significantly lower than in fed controls. That this weight differential resulted not from an actual loss in weight of the tumors in fasted animals, but rather from a greatly reduced rate of tumor growth, is demonstrated by Experiment V, in which data derived by comparison of tumor weights in the fasted mice with those of a group of tumor-bearing animals autopsied at the beginning of the fasting period, reveal minimal growth of the tumor during the fast. The failure of adrenalectomy to inhibit this retardation of tumor growth is in contrast to the previously observed inhibitory effect of adrenalectomy on the fasting involution of normal lymphoid tissue. As in the unoperated mice, the difference between mean tumor weight in fasted and fed adrenalectomized mice also appeared to result from a retardation of the rate of tumor growth rather than from an absolute reduction in the initial size of the tumor. No specific effect of adrenalectomy upon the weight or growth rate of the tumor in fed animals can be established from these data. It is of interest to note, however, that in a small group of fed, non-operated controls (Table I, Exp. XI), the mean tumor weight 13 days following implantation was twice the mean weight of tumor in fed, adrenalectomized mice of the same group. This retardation of growth rate cannot be attributed to a specific inhibitory effect of adrenalectomy, since the inanition succeeding adrenalectomy might suffice in itself to account for the data. It is also of interest that no consistently distinct changes in the composition of the tumor result from a 48-hour fast (Table II), in contrast to the depletion of lipid induced in thymus and mesenteric lymph nodes by a similar fast (Table II and below). Although the actual values at each interval are not reliable (as a result of considerable individual variation both in tumor weight and composition), data concerning the lipid composition of the tumor (Table II) during its growth in fed mice, indicate a consistently progressive depletion of lipid between the first and second week following implantation.

Thymus, lymph nodes and spleen. The involution of normal lymphoid structures during the course of a 48-hour fast in normal mice, and the dependence of this phenomenon on the presence of the adrenals(9,10) is also seen in the tumor-bearing mouse, although the magnitude of the involutional weight reduction on fasting is not uniformly as great as that established previously. The data of Table I indicate, in addition, a reduction in thymus-mesenteric node weight and an increase in spleen weight in fed tumor-bearing mice as compared with fed tumor-free controls. As is true of the tumor, a progressive depletion of lipid occurs in the thymus and mesenteric nodes of fed tumor-bearing mice as the tumor attains increasing age and mass (Table II). The remarkably high value of 15.9% lipid calculated for the thymus and nodes of fed mice in Experiment IV cannot be regarded as reliable, however, because of the high degree of individual variation in the analyses of this group. In contrast to the failure to demonstrate consistent depletion of lipid in the tumors of fasted animals, the characteristic depletion in lipid content in the thymus and mesenteric nodes induced by fasting(10), is maintained in the presence of the tumor. Adrenalectomized tumor-bearing mice, similarly, fail to mobilize fat from the thymus and mesenteric nodes during a fast to the same extent as is noted in fasted, unoperated controls.

Discussion. The striking retardation in the growth of a mouse lymphosarcoma resulting from a 48-hour fast indicates the dependence of this rapidly growing tissue on an undiminished exogenous source of energy and cell constituents. The persistence of its growth during the fast, albeit at a greatly reduced rate, and at the expense of the already depleted tissues of the fasting host, however, is consistent with the well-known autonomy of growth of malignant tumors. Unlike the physiologic involution of lymphoid tissue induced by the stress of a fasting period, this reduction in growth rate of the tumor is not apparently dependent on the maintenance of adrenal cortical secretions, an observation which demonstrates a distinct difference in behavior between the tumor and its morphologically homologous normal counterpart. However, other observations (2,3,4) have indicated that under some conditions malignant lymphoid tissue responds as does normal lymphoid tissue to certain endocrine stimuli. The persistence of the fasting involution of uninvolved lymphoid structures in lymphosarcoma-bearing mice, and its inhibition by adrenalectomy, serves to emphasize the difference in behavior between malignant and normal lymphoid tissue with respect to pituitary-adrenal cortical influence.

That the reduction in the mass of pooled thymus and mesenteric lymph nodes noted in fed, tumor-bearing mice may be adrenal-mediated is suggested by its failure to occur in the adrenalectomized animal, in which, indeed, the spleen attains a mass even greater than that noted in the presence of the tumor alone. It seems equally possible, however, that the presence of the tumor and adrenalectomy represent 2 independent influences on thymus-node mass with opposing effects, the resultant of which is exhibited in the preservation of normal lymphoid tissue weight.

Further data of interest relate to the relatively high lipid content of the tumor (and possibly of the pooled thymuses and mesenteric nodes of fed tumor-bearing animals), and the progressive decline of lipid concentration in these structures as the tumor attains increasing size in the fed animal. Whether this observation indicates a specific and important role of the tumor in the metabolism of lipid, either in the tumor tissue or in other tissues as well, and what its relation may be to the influence of the tumor in the production of fatty livers in fasted mice (7), is unknown at the present time.

Summary. Weight measurements and composition analyses of a transplantable lymphosarcoma and several normal lymphoid tissues in fasted, tumor-bearing CBA mice indicate that a 48-hour fast results in a marked retardation of tumor growth rate without completely inhibiting absolute growth. This tumor growth differential in fasted and fed mice is not dependent on the presence of the adrenals. On the other hand, uninvolved, non-malignant lymphoid structures in tumor-bearing animals show qualitatively the same involutional effect of fasting and the same dependence of this phenomenon on the presence of the adrenals as has been reported for tumor-free animals. Thus, malignant lymphoid cells (transplantable lymphosarcoma) in fasted mice exhibit a striking independence of the pituitary-adrenal cortical control which regulates the fasting involution of non-malignant lymphoid tissue. A considerably greater weight of the spleen was seen in fed, tumor inoculated mice, as compared to control animals. This splenic enlargement was apparent even at an early time following tumor inoculation when no tumor mass was palpable. The transplantable lymphosarcoma is relatively high in lipid content early in its development, but the percentage of lipid steadily declines as tumor size increases.

Mrs. Rita Huang contributed valuable assistance with the analytical and operative procedures.

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Effect of Beef Spleen Extract on the Respiration of Normal Mouse Tissues During Storage.* (18276)

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Previous communications(1,2) have dealt with the use of beef spleen extract(3) in the treatment of basal cell carcinoma and its mode of action(4,5). Earlier work from this laboratory(6), a study of the effect of beef spleen extract on the oxygen uptake of normal mouse liver as compared with that of dbrB carcinoma during storage at 4°C over a period of 12 days, suggested that the tumor was more vulnerable than the liver. In order to learn whether normal tissues differ among themselves and from tumorous tissue in their response, storage experiments were extended to the following normal tissues: brain, connective tissue, heart, kidney, liver and lung. The possible effects of bacterial contamination and of tissue breakdown on oxygen consumption during storage have been considered.

Experimental. Seventy-six female dba mice, 3 months old, were used as in the earlier work(6). Under light anesthesia and aseptic conditions the brain, connective tissue, heart, kidney, liver and lung were removed and macerated by passing through a fine mesh screen with a porcelain pestle. The pulp was suspended in twice its volume of Tyrode's solution and the suspension divided into 2 portions. To one was added one-third its volume of Tyrode's solution and to the other a similar volume of beef spleen extract. In the current experiments the beef spleen extract(3) contained 115 mg of solids per ml, the final concentration for storage being 30 mg per ml, and that during the period of measurement of respiration, 10 mg per ml. Respiration measurements were made in duplicate in Warburg respirometers at 37.5°C. The inner well of each respirometer flask contained 0.2 ml of 2 N sodium hydroxide and the outer well 2.5 ml of Ringer-phosphate-glucose solution (pH 7.2) and 0.5 ml of tissue suspension in Tyrode's solution or beef spleen extract. Preliminary experiments showed that the extract did not absorb oxygen. Readings were made after 60, 120, 180 minutes. The suspensions were then dried over-night in weighed crucibles in a drying oven at 70°C. The calculated weight of the solid content of the physiological solution was subtracted from the dry weight of the tissue. The cubic mm of oxygen per hour per mg of dry weight (Qo_2) of the first and second 60-minute intervals were calculated but only the total cubic mm of oxygen per 3 hours per mg of dry weight of tissue are tabulated. The values in the table represent the average of duplicate determinations which agreed within 10%.

With the foregoing technique respiration rates were determined at 48-hour intervals for each of the 6 tissues in beef spleen extract as well as in Tyrode's solution throughout the 18-day period of storage at 4°C. An initial determination of the oxygen consumption of the fresh tissue suspended in beef spleen extract and in Tyrode's solution was made within 30 minutes after its aseptic removal from the animal. These data are presented in the column of Table I designated as 0 day.

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† Unit of the Institutum Divi Thomae, Cincinnati, Ohio.

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TABLE I. Oxygen Consumption (cu mm O_2 per mg Dry Wt) Over 3 Hr Interval of Normal dba Mouse Tissues Stored 18 Days at 4°C in Tyrode's Solution and in Beef Spleen Extract.

	Lung		Heart		Kidney		Liver		Brain		Connective Tissue	
Days	Tyrode	Spleen	Tyrode	Spleen	Tyrode	Spleen	Tyrode	Spleen	Tyrode	Spleen	Tyrode	Spleen
0	2.03	0.68	2.50	1.73	6.23	8.04	3.09	2.56	12.53	3.64	.62	.53
2	2.31	1.99	1.37	2.17	2.88	6.99	2.24	2.37	4.28	3.00	.50	.84
4	1.03	1.18	1.18	0.42	2.93	5.72	3.21	2.95	4.95	1.42	.39	.22
6	0.92	0.88	0.78	0.44	3.05	4.18	2.05	1.57	1.68	1.04	.22	.12
8	0.49	0.58	0.28	0.34	1.23	2.25	1.10	1.36	1.63	1.03	.26	.10
10	0.66	0.48	1.26	3.98	1.37	2.08	1.15	1.27	1.10	0.90	.07	.10
12	0.62	4.48	1.65	7.08	2.53	2.08	1.31	2.00	1.42	0.88	1.02	.19
14	1.44	7.37	7.04	12.94	4.12	2.59	1.17	1.20	3.31	2.48	1.20	.19
16	2.53	11.63	7.20	22.07	6.64	3.53	0.95	1.04	6.36	3.53	3.51	.48
18	9.17	17.81			4.73	7.08	1.32	1.24	10.63	5.51	3.33	2.51

Microscopic examination of the tissue suspensions was made at 48-hour intervals, just a few minutes prior to making the respiration tests, for evidence of bacterial contamination. Loeffler's methylene blue and gentian violet were used for the stained preparations. Blocks of tissue stored simultaneously were fixed at the same time in 10 per cent formalin, embedded in paraffin, sectioned and stained with Harris' modification of Delafield's hematoxylin and eosin for histologic examination.

Results. In general, the respiration rate of all the normal tissues decreased on storage in both Tyrode's solution and spleen extract from the second to the eighth, tenth, or twelfth day. With the exception of liver, all tissues in both storage media thereafter exhibited an unexpected rise in respiratory rate (Table I). Examination of stained and unstained preparations of the stored tissues at the time of respiration tests for the presence of bacterial contamination revealed no changes in the types or relative numbers of organisms from those initially present and observed in the fresh aseptically removed tissues; an occasional member of the salmonella group was observed. Histologically there was no evidence of necrosis or cellular fragmentation during storage for this period. In a further attempt to determine whether tissue autolysis might have occurred during storage, with the production of oxidizable amino acids, the amino nitrogen was determined by the Van Slyke procedure at intervals during storage of the six tissues. Increases of only 0.3% to 5.4% were found, which were too small to account for the observed increase in oxygen uptake.

The immediate effect of beef spleen extract on the fresh unstored tissues, as shown in the tabulated data for 0 day, was marked depression of the amount of oxygen consumed by brain, lung, and heart tissue, a very slight decrease for liver and connective tissue, and an increased oxygen uptake by kidney. During the latter half of the storage interval oxygen consumption by lung and heart was greater in spleen extract than in Tyrode's solution. This was not noted in the case of kidney, brain and connective tissue which tended to have a lower respiration in the extract than in Tyrode's solution during this period. Liver tended to maintain a more or less steady respiration during the latter half of the storage period and it was relatively unaffected by the presence of beef spleen extract in confirmation of the earlier findings(6).

Discussion. The respiratory response of normal tissues to storage at 4°C for periods as long as 18 days in beef spleen extract varied. The values obtained for respiration of dbrB adenocarcinoma grown in dba mice under similar experimental conditions(6) fell within the extremes of the limits recorded in the present paper for the normal dba tissues. This is consonant with the observations of Greenstein(7) that the enzyme activities of

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7. Greenstein, J. P., Biochemistry of Cancer, Academic Press, Inc., New York, 1947, pp. 200-201.

tumors generally lie within the range for normal tissues, and likewise confirms the lack of specificity of spleen extract in its action on tumor tissues as reported by Fardon *et al.*(4).

The steady increase in respiration during the latter portion of storage in either Tyrode's solution or in beef spleen extract cannot be attributed to bacterial contamination nor, so far as the results of histologic examination and the determination of amino nitrogen show, to the autolysis of the stored tissues. It is possible that other oxidizable substrates may be released during storage.

Summary. By means of Warburg respirometers the oxygen consumption of brain, connective tissue, heart, kidney, liver and lung from normal dba mice was measured at 48-hour intervals during a period of 18 days of storage at 4°C, in Tyrode's solution and in beef spleen extract. The respiration of all six of the normal tissues, whether stored in Tyrode's solution or in beef spleen extract, decreased during the initial 8- to 10-day period, while the same tissues, with the exception of liver, were characterized by a progressive increase in respiration during the 12- to 18-day period of storage. This increase could not be correlated with bacterial contamination or with tissue autolysis as revealed by histologic observation and amino nitrogen determination. The immediate effect of the extract was to decrease the oxygen consumption of brain, lung and heart, and, to a lower degree, of liver and connective tissue, while that of kidney was increased. The effect of beef spleen extract on dbrB adenocarcinoma in dba mice fell between the extremes of the effects on normal dba mouse tissues.

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Activity of Some Enzymes Involved in Carbohydrate Metabolism in Normal and Scorbutic Guinea Pigs.* (18277)

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Previous work has shown that an ascorbic acid deficiency in guinea pigs causes a disturbance in carbohydrate metabolism manifested by a decrease in liver and body glycogen storage, high blood sugar levels, and lowered absorption after glucose ingestion(1,2). When deficient animals were treated with adrenal cortical extract, insulin and adrenaline no improvement resulted(3). This suggests the possibility that the disturbance may be enzymatic rather than hormonal in origin.

The following study was designed to investigate the activity of several enzymes involved in carbohydrate metabolism in both normal and deficient animals. Phosphorylation and oxidation in kidney and liver extracts were first measured. Ochoa(4) found that even in the presence of fluoride, adenosine triphosphatase interfered with the phosphorylation of glucose by adenosine triphosphate and with the transfer of phosphate from phosphocreatine to glucose. This suggests the possibility that the lowered utilization of glucose in the scorbutic animal may be due to a disturbance in phosphorylation due to increased activity of adenosine triphosphatase.

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4. Ochoa, S., *J. Biol. Chem.*, 1943, v151, 493.

Experimental. Young guinea pigs (550-650 g) were paired as to sex and weight. They were pair-fed and maintained on a commercial rabbit food.† In addition to the diet all animals received a carotene-in-oil supplement sufficient to supply 1 mg of carotene daily. The normal animals were given a solution containing 10 mg of ascorbic acid 3 times weekly.

Animals kept on this diet without ascorbic acid supplementation exhibited a decreased appetite in 19-24 days. At this stage scurvy was present as evidenced by extensive hemorrhages of the fascia of the musculature particularly the legs. A rapid weight loss accompanied the loss of appetite. A few days after inanition became apparent in the deficient member of the pair, both animals were fasted 24 hours, anesthetized with sodium amytal (sodium iso-amyl ethyl barbiturate) and killed by exsanguination. Liver and kidneys were removed and placed immediately in ice cold Ringer's solution. After chilling, the liver was cut in small pieces, 10 g weighed and homogenized in a test tube (using a power driven pestle as developed by Potter and Elvehjem)(5) with 1.2 ml of 0.1 M phosphate buffer pH 7.7 per g of tissue. Both kidneys were cut up, weighed, and prepared as was the liver with the exception that 2.4 volumes of phosphate buffer were added for each gram of tissue. Liver homogenates were centrifuged for 10 minutes and kidney for 5 at approximately 2000 revolutions per minute‡ and the supernatant decanted for use. Microscopic examination of the extract showed some cell fragments. Meyerhof and Geliazkowa(6) pointed out that the enzymic extracts are less complex systems than homogenates and give more uniform results, therefore extracts were used. All extracts and flasks were chilled until placed in water bath. The technique of Colowick, Welch and Cori(7) and Colowick, Kalckar and Cori(8) was used for the determination of phosphorylation and respiration. Respiration determinations were made in air at 37°C in a Warburg respirometer on duplicate samples. Substrates (Table I) were added to the side arm of the respiration flasks; 1 ml of tissue extract and 0.1 ml of 4% sodium fluoride were placed in the body of the flask and 0.2 ml, 20% NaOH and filter paper in center well. Samples were equilibrated for 10 minutes, the vessels closed, substrates dumped from the side arm and readings taken every 15 minutes. Liver samples were incubated for 60 minutes but kidney was run only 30 minutes since respiration was so rapid readings could not be taken after this time. Q_{O_2} was calculated using the dry weight of 1 ml of extract corrected for the salt content of the buffer. After incubation samples were made to a volume of 30 ml with distilled water, 1 ml of this made to 10 with 10% trichloracetic acid, centrifuged, and inorganic phosphate determined on the supernatant by the method of Fiske and Subbarow(9). Blanks were secured by determining the phosphate in 1 ml of the original extract diluted and treated as above. Phosphorylation was calculated by the difference between blank and incubated sample. Adenosine triphosphatase was determined by the method of Dubois and Potter(10) modified by the use of a borate-KCl buffer in place of diethyl barbiturate. One gram of liver was homogenized with 9 ml of water; 1 ml of this homogenate made to 10 m with water. The diluted homogenate (0.4 ml) was placed in the side arm of a Warburg vessel; 0.3 ml of 0.03 M borate-KCl buffer (pH 7.7), 0.1 ml of 0.04 M calcium chloride and 0.6 ml of

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† Centennial Rabbit Grain Pellets.

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‡ Radius of centrifuge head to center of fluid 7 cm; horizontal head used.

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TABLE I. Phosphorylation, Oxidation, Phosphorylase, Adenosine Triphosphatase and Aldolase Determinations in Tissues of Normal and Scorbutic Guinea Pigs.

Phosphorylation and oxidation—1 ml extract, 2.9 micromoles adenylic acid, 1.2 micromoles cozymase, 60 micromoles glucose, 30 micromoles glutamic acid, 60 micromoles succinic acid, 10 micromoles $MgSO_4$, 95 micromoles NaF. Total vol. 1.9 ml.

Animals	No.	Phosphate esterified, mg Liver	Phosphate esterified, mg Kidney	Q_{O_2} Liver	Q_{O_2} Kidney
Normal	9	.56	.73	1.37	8.77
Deficient	10	.16	.59	2.40	8.57

Adenosine triphosphatase determinations

Animals	No.	Phosphate liberated from liver extract, mg
Normal	10	.015
Deficient	9	.021

Phosphorylase determinations

1 ml extract, 2.9 micromoles adenylic acid, 30 micromoles glucose-1-PO_4 (.88 mg acid labile PO_4), 4 micromoles glycogen. Total vol. 2 ml.

		Phosphate liberated, mg					Glycogen* formed, mg	
Animals	No.	Liver total*	7 min.	Kidney total	7 min.	Muscle total	Liver	Kidney
Normal	8	.80		.71		.25		
Deficient	8	.53		.59		.33		
Normal	10	.59	.18	.57	.29		24.1	28.8
Deficient	10	.59	.22	.45	.18		30.9	25.3

Aldolase determinations

1 ml extract, 0.012 micromoles fructose-1, 6-diphosphate, 1 ml 0.1 M glycine buffer, 0.25 ml hydrazine sulfate.

Colorimeter readings

Animals	No.	Liver	Kidney	Muscle
Normal	5	58	71	71
Deficient	5	65	70	69

* Theoretical yield phosphate 1.33 mg, glycogen 26 mg.

adenosine triphosphate (0.7 micro-mole containing 0.04 mg acid labile P) was added to the body. Flasks were equilibrated for 10 minutes in a water bath at 37°C after which homogenate was dumped into the contents in the body and the whole incubated for 15 minutes. The flasks were removed and 0.6 ml of 10% trichloracetic acid added; the contents removed and centrifuged. Phosphate was determined on the supernatant fluid.

Phosphorylase activity in liver, kidney and muscle was determined according to the method of Cori, Cori and Green(11) by measuring phosphate liberated and glycogen formed according to the reaction

$$\text{glucose-1-}PO_4 \xrightarrow{\text{phosphorylase}} \text{glycogen} + PO_4$$

Liver and kidney extracts were prepared as were those used to measure phosphorylation with the substitution of a 0.015 M cysteine for the phosphate buffer. Muscle was weighed and ground in sand with 2.4 volumes of the buffer. Tissue extract was added to the side arm and substrate (Table I) placed in the flask. Flasks were equilibrated for 10 minutes in the water bath, dumped and incubated for one hour with free access to air at 37°C. Samples were then diluted as for phosphorylation measurements and inorganic phosphate determined.

Since glucose-1-PO_4 can be converted to glucose-6-PO_4 or glycogen, another series of animals were run and glycogen as well as phosphate and 7 minute phosphate determined on incubated samples. Glycogen was determined by the method of Good, Kramer

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and Somogyi(12). Aldolase activity in liver, kidney and muscle from normal and scorbutic animals was also investigated. Aldolase catalyzes the reaction

$$\text{fructose-1,6-diphosphate} \xrightarrow{\text{aldolase}} \text{glyceraldehyde-3-}PO_4$$

The method of Sibley and Lehninger(13) was used. Liver, kidney and muscle were ground with two times their weight of Ringer's solution. One ml of the trichloracetic acid extract resulting from the procedure was diluted to 10 ml with distilled water and 1 ml used for colorimeter readings.

Results and Discussion. Results of the analytical work are shown in Table I. When total phosphorylation was measured, the liver tissue of the normal animal showed a markedly greater ability to phosphorylate substrates than tissue from the deficient animal. The difference in kidney tissue was much less marked. Since crude extracts were employed the activity of several enzymes must have been measured. Colowick, Welch and Cori(7) state that the product of aerobic phosphorylation of glucose in liver extracts containing sodium fluoride was mainly fructose diphosphate. In some instances these authors using kidney extracts found a considerable proportion of the product of glucose phosphorylation to be phosphoglyceric acid. They state that succinic acid oxidation caused phosphorylation of glucose; this means that the dehydrogenation of succinic acid to fumaric is connected with the transfer of inorganic phosphate to glucose.

The oxygen consumption of liver tissue in animals of both groups was relatively constant but kidney gave variable results. Oxygen consumption of liver increases noticeably in scurvy but there is no change in kidney. These results are in agreement with the findings of Stotz, Harrer, Schultze and King(14).

Adenosine triphosphatase activity of liver tissue was higher in the deficient animal as measured by the liberation of phosphate from adenosine triphosphate. This may indicate hyperactivity of this enzyme resulting in reduced amounts of this compound.

Phosphorylase measurements in the first group of normal animals gave higher results for both liver and kidney extracts. This was not true of muscle extracts. In the second series the results for liver were the same but a higher figure was again obtained in kidney extracts from normal animals. Glycogen formation in liver and kidney extracts from the two groups of animals was about the same. Apparently the inability of the scorbutic animal to form glycogen as efficiently as the normal cannot be due entirely to reduced phosphorylase activity.

There appeared to be no difference in aldolase activity in liver, kidney and muscle tissue from the two groups of animals.

Summary. Liver tissue of normal guinea pigs showed a markedly greater ability to phosphorylate substrates than tissue from ascorbic acid deficient animals. The difference in kidney tissue was less marked. The oxygen consumption of liver tissue increases noticeably in scurvy but there is no change in kidney. Adenosine triphosphatase activity of liver tissue was higher in the deficient animal as measured by the liberation of phosphate from adenosine triphosphate. Phosphorylase measurements in one group of normal animals gave higher results for both kidney and liver extracts. This was not true of muscle extracts. In the second series the results for liver were the same but a higher figure was again obtained for kidney extracts from normal animals. Aldolase activity did not seem to be lowered in scurvy.

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Prevention of Postoperative Adhesions in Rabbits with Streptococcal Metabolites.* (18278)

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In the past, investigators have used various drugs in attempts to prevent postoperative adhesions, but without success. Rea and Wangensteen(1) introduced defibrinated ox blood, amniotic fluid, sodium ricinoleate, ether and normal saline into the peritoneal cavity; Totten(2) used hypertonic glucose; Merkle (3) and Gepfert(4) instilled amniotic fluid intra-abdominally. Ochsner and Garside(5), Donaldson(6), and Ward(7) tried proteolytic enzymes such as papain to digest deposits of fibrin. The work of Lehman and Boys(8), and Van Dyk(9) have suggested that the incidence and extent of postoperative adhesions may be reduced by the introduction of heparin into the peritoneal cavity at the time of operation and in the early postoperative period. However, Bloor and his associates(10) reported a series of experiments in rabbits in which they concluded that heparin did not prevent the formation or reformation of intraperitoneal adhesions or the deposition of fibrin. In the investigations of White(11), heparin and dicumarol appeared to be effective in decreasing but not preventing postoperative peritoneal adhesions in dogs. The method by which healing and hence adhesion formation takes place is well known and will not be reviewed here. Suffice it to say that fibrin must be present before scar tissue can be formed. The amount of fibrin formed varies with the irritant and with the individual. In serous cavities fibrinous inflammation is common. In all types of fibrinous inflammation, variable numbers of polymorphonuclear leucocytes are entangled in the threads of fibrin. Sherry *et al.*(12) have accumulated evidence that a considerable amount of nucleo-protein, which is not fibrin or its denaturation product, is present in inflammatory exudates. Tillett, Sherry and Christensen(12) have indicated in their reports that desoxyribose nucleoprotein, together with deposits of fibrin, account for the gelatinous and stringy coagulum present in purulent exudates.

It has been demonstrated that some hemolytic streptococci elaborate, during their growth in broth cultures, both a fibrinolytic principle (streptokinase) and a potent desoxyribonuclease, (streptodornase)(12). When streptococcal concentrates are added to whole samples of inflammatory exudates *in vitro*, the rapid lysis of both fibrin and desoxyribose nucleoprotein occurs. Because of this action on fibrin and inflammatory exudates, a logical application for these streptococcal metabolites presented itself, namely, in experimental postoperative adhesions.

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* The Streptokinase and Streptodornase used in these studies were supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

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TABLE I. Summary of Results with Streptokinase-Streptodornase in the Prevention of Postoperative Adhesions in Rabbits.

			Results		
Animals	No. of rabbits	Form of drug	No. with adhesions to scarified area	No. with adhesions to infected sutures	No. with no adhesions
Control	25	0	23	6	2
Treated	6	Powder	0	6	0
	19	Solution	0	4	15

Materials and methods. Fifty male rabbits, weighing from 2½ to 3½ kg were used in these experiments. They were divided into a control and an experimental group of 25 animals each. The latter group received streptokinase and streptodornase intraperitoneally at the time of operation. In all groups, surgery was performed under intravenous sodium nembutal anesthesia. A midline incision was made and an area of peritoneal wall, measuring 1 x 2 inches, on either side of the midline, was abraded with a scarifier. The scarifier was made by cutting a one-inch square from a large vegetable grater. The scarification procedure was standardized by using five strokes per area. Slight oozing of the abraded areas was produced intentionally in all cases. In one group of 8 control and 8 experimental animals, segments of the ileum and colon were also scarified, but ample adhesions were found to result simply from the abrasions of the peritoneal wall. The latter procedure was carried out in all subsequent experiments. Streptokinase and streptodornase were both contained in the same preparation and the dosage used varied from 600,000 to 1,400,000 streptokinase units per animal and from 180,000 to 350,000 streptodornase units per animal. The first 6 animals received the powdered drug, whereas in the remaining nineteen animals the drug was diluted in 10 cc of normal saline before instillation. In this series, the drug was administered intra-abdominally only at the time of operation. A laparotomy was performed on all animals 14 days after the initial operation and the number and extent of the adhesions were noted.

Experimental results. Fifty rabbits survived operation and scarification. A summary of the results is shown in Table I. Twenty-three control animals developed extensive adhesions to the areas of scarifications and to the suture line. A typical result is shown in Fig. 1. In the treated group, the 6 rabbits which received powdered streptokinase and streptodornase had been repaired with through and through silk sutures and all of these animals developed adhesions, varying from slight to dense, to the infected suture line. All of the remaining treated animals received the drug in solution and were closed in layers. Four animals in this group had slight to medium adhesions to the infected suture line, while 15 had no adhesions at all. None of the treated animals developed adhesions to the area of scarification. All of the control animals with adhesions had them at the scarification site. In each case the abraded areas of the treated animals had healed within the observation period (Fig. 2). It is interesting to note that the animal receiving the highest dosage of drug, 1,400,000 units of streptokinase and 350,000 units of streptodornase, failed to form adhesions at the incision even though there was a massive infection of the suture line. No signs of irritation or toxicity due to the drug were seen in any of the treated animals.

The results of these experiments demonstrate rather conclusively the usefulness of

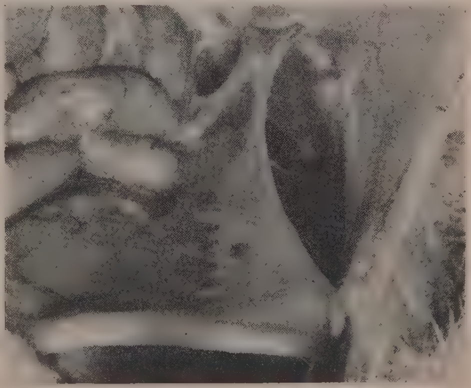

FIG. 1.
Control animal showing omentum adherent to site of scarification.

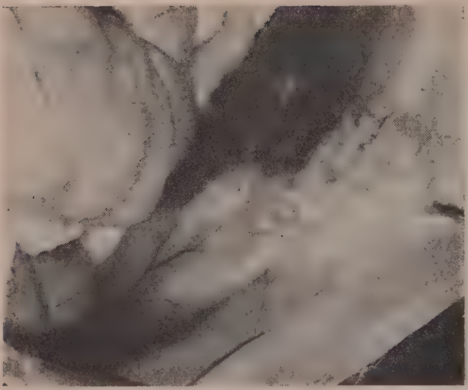

FIG. 2.
Treated animal showing no adhesions to site of scarification.

streptokinase and streptodornase for preventing postoperative intra-abdominal adhesions in rabbits due to trauma. It should be emphasized that in all of the treated animals with adhesions, the bands were adherent to the infected suture line, not to the area of scarification. The last series of experiments, in which no infection was found at the suture line, revealed no adhesions in the treated animals. Of the 10 treated rabbits with adhesions, 6 were closed with through and through sutures, and all of these animals had extensive infections at their suture lines. Inasmuch as the drug was administered only at the time of operation, it is conceivable that the enzymes were no longer active as the relatively slowly progressing infection extended from the suture line. It is interesting to note that wound cultures taken from infected suture lines revealed predominantly fecal organisms. In the present report, the optimum dosage was not determined. However, the results were found to be much better when the drug was placed in solution prior to use than when used in the dry form.

Conclusion. The results in this series of animals were sufficiently encouraging as to justify the further investigation of streptokinase-streptodornase in the prevention of experimental postoperative adhesions.

Addendum: Preliminary work in dogs has indicated so far that the drug does not prevent the formation of postoperative intra-abdominal adhesions in this animal. This may be due, however, to a variety of factors such as insufficient dosage, animal inactivators or inability of streptokinase and streptodornase to activate dog's serum. Kiser's(13) experiments have shown that *in vitro,* streptokinase and streptodornase fail to activate dog's serum except in very high dosage.

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Effects of a Transient Vitamin A Deficiency on Subsequent Resistance to Cold.* (18279)

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It is becoming increasingly apparent that malnutrition may cause residual effects that persist long after dietary deficiencies have been corrected and the gross manifestations of nutritional disease have been ameliorated (1). Recent findings by Best and Hartroft (2) are pertinent in this regard. These workers found that less than a week of deficiency of choline or its precursors in the diets of young rats led to high blood pressure in 25% of the animals maintained on a good normal diet except for this short interval. Similar findings have been reported by others(3). The irreversible testicular degeneration of male rats fed a vit. E-deficient diet is another example of a residual effect of malnutrition. In the present communication data are presented concerning the residual effects of a transient vit. A deficiency in rats on the resistance of these animals to subsequent exposure to cold.

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Communication No. 274 from the Department of Biochemistry and Nutrition, University of Southern California.

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Procedure and results. The animals employed in the present experiment were male rats of the University of Southern California strain in which the dietary regime of the mothers was such as to make the young rats satisfactory for bioassay studies for vit. A. The mother rats were maintained for at least 2 months previous to breeding and for 12 days after the birth of the litter on Sherman diet B (4) without addition of supplementary lettuce or meat. Litters were cut to 7 at 3 days of age, and on the 12th day mothers and litters were placed on a vit. A-low diet.† The young were weaned at 21-24 days of age and at a body weight of 40-50 g inclusive. At this time the young were divided into 3 groups. All animals were fed *ad lib.* a vit. A-free diet (U.S.P. XIII, to which were added 75 mg of mixed natural tocopherol esters per kg of diet), but they differed in the following respects. Group I received 6 days weekly a supplement of 50 U.S.P. units of vit. A.‡ These rats did not plateau in body weight and they showed no symptoms of vitamin A deficiency at any time during the experiment. Groups II and III were fed the same diet as Group I but the vitamin A supplement was withheld. These animals depleted of vit. A after 20 to 22 days of feeding at which time their body weight averaged approximately 105 g. The end of the depletion period was set as the 5th day on which rats on the vit. A-free diet showed stationary or decreasing weight. After depletion the animals in Group II received the same vitamin supplement as those in Group I (50 U.S.P. units of vit. A per day, 6 times weekly). Animals in this group gained weight rapidly following the vit. A administration, which was continued for a period of 12 weeks. Rats in Group III differed from those in Group II in the dosage of vit. A which was administered after depletion. For the first 28 days after depletion animals in Group III received 6 times weekly a supplement of 1.25 to 2.25 U.S.P. units of vit. A acetate per day. The average gain in body weight of rats in this group during this period was 47 g. Subsequent to the 28th day of feeding the above suboptimal dosage of vit. A, and for 8 weeks thereafter, animals in this group were administered the same vit. A supplement that was employed for Groups I and II. Twelve weeks after depletion rats in Groups II and III averaged 255 g and 241 g respectively in contrast to an average body weight of 269 g for animals of a similar age in Group I. No significant difference in gross appearance was observed between animals in any of the 3 groups.

After the 12-week refeeding period 24 rats each of Groups I and II and 22 rats of Group III were selected for further study. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C and (2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. Six rats of each group were employed in the room temperature series; the balance in the cold room series. Animals were kept in individual metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib.* the following purified diet: sucrose, 60%; casein,§

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† Similar to U.S.P. XIII depletion diet except that commercial casein rather than extracted (vitamin A-free) casein was employed.

‡ Natural ester concentrate from Soupfin shark liver oil, prepared by Collett-Week-Nibecker, Ossining, N. Y. 50 U.S.P. units of Vit. A Natural Esters were dissolved in 0.1 ml of cottonseed oil containing 0.5% alpha-tocopherol as an antioxidant.

TABLE I. Effects of a Transient Vitamin A Deficiency on Subsequent Resistance to Cold.

Group	No. of animals	Initial wt, g	Change in body wt over 50-day period* g	% decedents	Avg survival time of decedents*
			Cold Room Series		
I	18	276.4	— 34.3 ± 3.6 (17)	5.6	38.0 ± 0.0
II	18	255.6	— 28.5 ± 5.2 (12)	33.3	21.3 ± 5.7
III	16	239.8	— 21.0 ± 5.4 (5)	68.8	28.8 ± 4.2
			Room temperature series		
I	6	254.1	+ 88.2 ± 10.9 (6)	0	—
II	6	254.8	+115.2 ± 8.6 (6)	0	—
III	6	247.6	+ 79.6 ± 9.7 (6)	0	—

The values in parentheses indicate the number of animals which survived and on which averages are based.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

25%; salt mixture,[||] 5%; and cottonseed oil (Wesson), 10%. To each kg of the above were added the following synthetic vitamins: thiamine hydrochloride, 40 mg; riboflavin, 40 mg; pyridoxine hydrochloride, 40 mg; calcium pantothenate, 80 mg; nicotinic acid, 60 mg; ascorbic acid, 100 mg; biotin, 4 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12},[¶] 100 μg; 2-methylnaphthoquinone, 10 mg; and choline chloride, 2 g. Each rat also received 6 times weekly a vit. A-D concentrate[**] containing 50 U.S.P. units of vit. A and 5 U.S.P. units of vit. D, and 3 times weekly a supplement of 1.5 mg alpha-tocopherol acetate. Feeding was continued for 50 days or until death, whichever occurred sooner.

Results are summarized in Table I. Findings indicate that a transient sub-lethal deficiency of vit. A markedly impaired the rats' ability to withstand subsequent exposure to a low environmental temperature. In the room temperature series all animals survived the experimental period of 50 days; in the cold room series, however, a significant difference was observed between the various groups in the ability of rats to withstand prolonged exposure to cold. Whereas only 1 out of 18 rats which had received vitamin A continuously from weaning on succumbed under cold room conditions, 17 out of 34 rats which had previously been depleted of vit. A (Groups II and III) and subsequently refed with this vitamin died under similar conditions. The mortality rate was particularly marked for animals in group III which had been depleted of vit. A and fed for 28 days thereafter a suboptimal amount of this vitamin. Values for Group II were intermediate between those for Groups I and III. After the third week of cold exposure necrotic changes were observed on the paws and tip of the tail of virtually all rats in the cold room series. Amputation of part or all of the tail occurred in approximately one-fourth of the series. A number of animals developed priapism. At autopsy kidney, adrenal and ventricular weights were sig-

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[§] Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

[||] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

[¶] Vit. B_{12} Oral Grade Solids, Chas. Pfizer & Co., Brooklyn, N. Y.

[**] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vit. A and 80,000 U.S.P. units of vit. D per g.

nificantly increased and testes weight significantly reduced over that of room temperature controls. In general, however, no significant difference in body weight or gross appearance was observed between the various groups either under cold room or room temperature conditions.

Discussion. Available data indicate that requirements for a number of nutrients are markedly increased under conditions of low environmental temperature. This is particularly true for some of the water-soluble vitamins. An increased requirement for thiamine(5,6), pyridoxine(7), and ascorbic acid (8) has been demonstrated following prolonged exposure to low environmental temperatures. Requirements are also increased for vit. A(9). Previous findings indicate that the survival time of vit. A-deficient rats is markedly reduced under conditions of low environmental temperature(9). Results of the present experiment indicate that a transient pre-test deficiency of vit. A (apparently corrected by the administration of relatively high doses of vit. A) may also impair the ability of rats to withstand subsequent exposure to cold. Available data, however, do not indicate what factors are responsible for the lowered resistance.

Findings of the present experiment suggest that transient nutritional deficiencies may leave residual effects in the organism which normally are not readily apparent but which may become manifest under conditions of stress. Further experiments are indicated to determine the residual effects of transient nutritional deficiencies (of other nutrients as well as vitamin A) and the aggravating effect of stress factors thereon.

Summary. A transient deficiency of vit. A, apparently corrected by the administration of relatively high doses of this vitamin, markedly impaired the ability of rats to withstand subsequent exposure to cold.

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Effects of Atropine on Brain Respiration.* (18280)

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The pronounced central nervous disturbances of atropine poisoning and the use of the drug in the treatment of postencephalitic Parkinsonism suggest the desirability of an examination of its metabolic action on central nervous tissues. It has been shown *in vivo*(1) that atropine depresses the oxygen consumption of brain. *In vitro* experiments on the effects of atropine on other tissues, *e.g.* muscle(2), have produced negative results. However, data on the effects of the alkaloid on the *in vitro* respiration of brain homogenates are lacking. This paper describes some effects of atropine on the respiration of rat brain homogenates.

Methods. Rats of mixed strains were used. After decapitation the brains were removed, weighed and homogenized in glass homogenizers(3). The homogenates were made directly in the complete medium used, the only

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TABLE I. Effect of Atropine on Endogenous Respiration in Dilute Buffered Saline.

pH	Drug	Q_{O_2}	% inhibition
6.0	—	4.8	—
	ACh*	4.64	3.3
	At†	4.66	2.9
	AA‡	4.33	6.6
	—	2.99	—
	At	2.89	3.3
7.0	—	3.03	—
	At	2.69	11.1
	—	4.85	—
	At	4.30	11.3
8.0	—	5.43	—
	ACh	5.39	0.7
	At	3.77	30.6
	AA	3.69	32.1
	—	3.02	—
	At	1.60	47.0

Medium. 0.033 M phosphate buffer; NaCl to give ionic strength 0.2. Added drugs: * acetylcholine-0.01 M; † atropine sulfate-0.001 M; ‡ acetylcholine chloride-0.01 M together with atropine sulfate-0.001 M. All concentrations refer to final concentrations.

further addition being the desired amount of alkaloid. The media were dilute buffered saline (Table I), Krebs-Ringer phosphate(4) and Robinson's medium(5). Respiration of the homogenates was measured in the standard Warburg apparatus at 37.7°C. The gas phase in all cases was air. Readings were commenced after a 5-minute equilibration period and were made at 10-minute intervals over periods of 90 to 120 minutes. The spectrophotometric data were obtained using the Beckman quartz spectrophotometer.

Results. Fig. 1 and 2 show clearly the inhibitory action of atropine and the partial reversal of the inhibition by acetylcholine. It should be noted that the data from which these curves were constructed were obtained under conditions of complete media with respect to inorganic ions and with glucose as substrate.

The effects of pH and acetylcholine addition (Table I) were particularly interesting in the light of experiments on invertase inhibition by atropine(6). In the case of invertase a similar progressive increase in inhibitory action with increasing pH was observed.

A study of the absorption spectrum of atropine under the same conditions of pH and ionic strength as prevailed in the respiration experiments mentioned above showed successive increases in optical density with pH in the position of the first maximum at 245-49 mμ (Fig. 3). It will be noted that the increment for the step in pH from 7 to 8 was greater than that for the preceding pH increase from 6 to 7. Similarly, there was a greater increase in inhibition of brain respiration corresponding to the first mentioned pH increase than for the latter. The significance of this is not clear.

The effects of acetylcholine in the case of the dilute buffered saline in the absence of substrate were either absent or slightly depressant. Although no systematic study of ionic effects has been

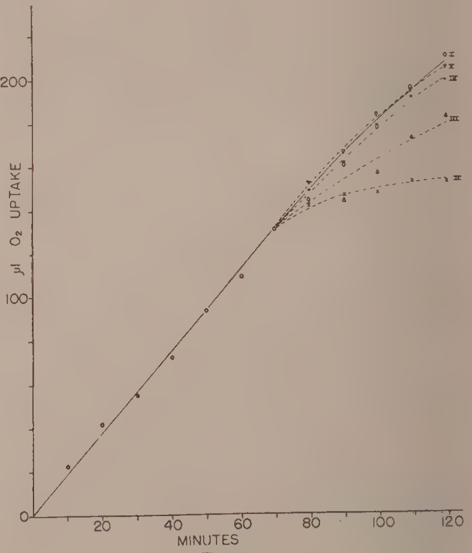

FIG. 1

The inhibition of respiration by atropine and partial reversal of the inhibition by acetylcholine. System: 10% homogenates of whole brain in Robinson's medium (1949). Additions of atropine sulfate or acetylcholine chloride made at 70 min. I. Normals; II. 10^{-2} M atropine added; III. 10^{-3} M atropine added; IV. 10^{-2} M atropine and 10^{-2} M acetylcholine added together; V-10^{-3} M atropine and 10^{-2} M acetylcholine added together. 37.7°C, pH 7.4. All concentrations refer to final concentrations.

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4. Krebs, H. A. and Henseleit, K., *Z. Physiol. Chem.*, 1932, v210, 33.

5. Robinson, J. R., *Biochem. J.*, 1949, v45, 68.

6. Rona, P., van Eweyk, G. and Tennenbaum, M., *Biochem. Z.*, 1924, v186, 276.

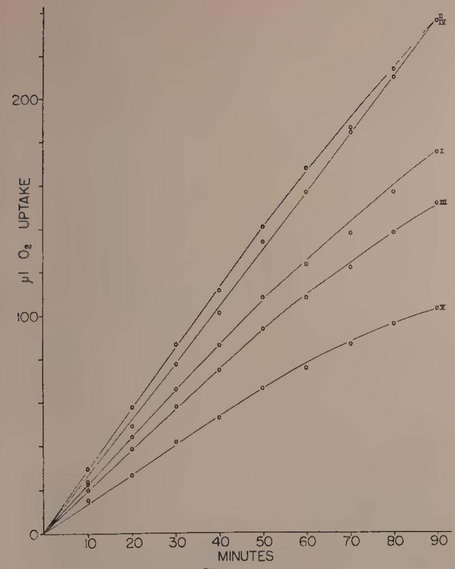

FIG. 2.

Effects of atropine and acetylcholine on eserinized homogenates. System: 10% homogenates of whole brain in Robinson's medium (containing glucose) incubated with acetylcholine chloride, atropine sulfate, and physostigmine salicylate before readings were made. I. No addition of drugs; II. 10^{-4} M physostigmine added; III. 10^{-2} M atropine, 10^{-2} M acetylcholine, and 10^{-4} M physostigmine added together; IV. 10^{-2} M acetylcholine and 10^{-4} M physostigmine added together; V. 10^{-2} M atropine only added. 37.7°C, pH 7.4. All concentrations refer to final concentrations.

carried out, some additional data appear in Table II which, considered with the previously presented data, seemed to indicate that the potassium ion was important in the acetylcholine reversal effect. Apparently an added substrate was also necessary for acetylcholine reversal. Incomplete as these results are they suggest that there is a considerable degree of complexity in the factors influencing the inhibition of respiration by atropine.

The stimulatory effect of physostigmine (Fig. 2) was also noted in washed homogenates with pyruvate as a substrate (unpublished).

Since added acetylcholine in the absence of a cholinesterase inhibitor failed to affect oxygen consumption, it seemed unlikely that the oxidation of products of acetylcholine hydrolysis was responsible for the reversal effect.

Discussion. In addition to the rather weak inhibitory action of atropine on brain respiration a point of particular interest was the observation of an atropine-acetylcholine antagonism in a metabolic sense and the possible significance of this antagonism in a system lacking structural integrity. It is difficult to explain the actions of these drugs in this case on the basis of competition for 'receptors' when the term is used with any implied or explicit morphological meaning(7). These results are of interest regarding Welsh's(8) suggestion that acetylcholine may function as a co-factor in some enzymatic process.

The pH dependence of atropine inhibition is clearly indicative of the unionized amine being the inhibitory form of the molecule. Much additional information is required to

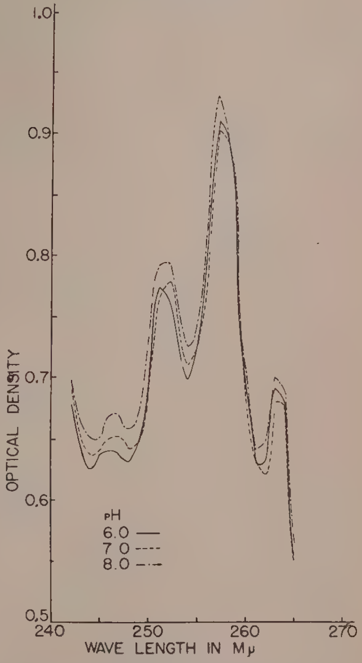

FIG. 3.

The absorption spectrum of atropine sulfate as a function of pH. Solutions of atropine were made in 0.033M phosphate buffers containing NaCl to make the ionic strength 0.2. Blanks were the corresponding saline buffers without atropine. The concentration of atropine was 10^{-2} M.

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7. Marrazzi, A. S., *Bull. Johns Hopkins Hosp.*, 1948, v83, 580.

8. Welsh, J. H., *ibid.*, 568.

TABLE II. Effect of Atropine on respiration supported by Alpha-glycerophosphate in Various Media.

Medium	Drug	% inhibition
I	10^{-3} M At*	41
II	10^{-2} M At	23
	10^{-2} M AA†	7
III	10^{-3} M At	15
	10^{-2} M At	41

Medium I. Ca-free Krebs-Ringer Phosphate with NaCl replaced by KCl; medium II. Krebs-Ringer Phosphate, Ca-free; medium III. Robinson's medium (see ref. 4) (similar to Krebs-Ringer but with somewhat higher buffer concentration). * Atropine sulfate; † Equimolar atropine sulfate and acetylcholine chloride together. Alpha-glycerophosphate was 0.01 M in all cases. All concentrations refer to final concentrations in the test system.

relate the spectroscopic changes to the ionization and corresponding respiration inhibition if such a relationship exists between all 3 factors. An obvious difficulty is the requisite demonstration of the manner in which ionization of the amine moiety affects the tropic acid group which is chiefly responsible for the absorption characteristics of the whole molecule(9). The importance of the ionic state of inhibitors has been emphasized in studies on cyanide and azide(10,11,12) and on certain antiseptics(13).

Summary. (1) It was found that atropine in relatively high concentrations inhibited the respiration of rat brain homogenates by as much as 40% under optimal conditions. (2) The respiration inhibition was partially reversible by acetylcholine under certain conditions. (3) The inhibition was pH dependent, increasing with increasing pH. Corresponding changes in the atropine absorption spectrum with varying pH were observed.

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9. Fischer, H., *Arch. exp. Path. Pharm.*, 1933, v170, 623.

10. Horecker, B. L. and Kornberg, A., *J. Biol. Chem.*, 1946, v165, 11.

11. Horecker, B. L. and Stannard, J. N., *J. Biol. Chem.*, 1948, v172, 589.

12. Stannard, J. N. and Horecker, B. L., *ibid.*, 599.

13. Simon, E. W. and Blackman, G. E., *Symp. Soc. Exp. Biol.*, 1949, v3, 253, Cambridge.

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The Growth-Promoting Action of Various Supplements in the Hyperthyroid Rat.* (18281)

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Studies on the deficiencies of rations composed principally of corn and soy-bean-oil meal for normal reproduction and lactation in rats and swine have been in progress at this station for several years. In the course of these investigations, it was deemed desirable to undertake the development of an assay procedure that was less time-consuming and more sensitive than that involving reproduction. One of the procedures used by other workers has been assessment of the ability of a supplement to reverse the growth inhibition induced by feeding thyroid-active agents to the immature rat. In the presence of an ample supply of other nutrients, it has recently been shown by Emerson(1), by Betheil and Lardy(2),

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* Published with the approval of the Director, Oklahoma Agricultural Experiment Station. Appreciation is expressed to Merck and Co., Rahway, N. J., for supplies of crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for pterolylglutamic acid and APF concentrates; to Armour and Company, Chicago, Ill., for liver fractions; to U. S. Industrial Chemicals, New York, for DL-methionine, and to Cerophyl Laboratories, Kansas City, Mo., for iodinated casein.

1. Emerson, G. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 392.

2. Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, v37, 495.

and by Frost *et al.*(3) that the administration of thyroid-active substances will induce a retardation in growth which is partially overcome by the supplementation of the ration with vit. B_{12}. Employing a somewhat different type of ration containing desiccated thyroid, however, Ershoff(4) was unable to demonstrate any positive effect of crystalline B_{12} on growth but found that whole liver substance and various liver fractions were highly active. The basal ration employed in the studies reported here was designed to resemble as nearly as possible the corn-soybean meal ration used in previous reproduction studies (Watts *et al.*(5)) and yet be composed of materials of more uniform quality. The ration developed, therefore, used isolated soybean protein and corn starch to replace natural feedstuffs. In view of the recent suggestion by Lewis, *et al.*(6) that the variation in results obtained by different groups of workers may be due to the carbohydrate component of the ration, the results obtained with this ration, which differs in both source of protein and source of carbohydrate from that used by others, are of additional interest.

Experimental. Rats used in all these studies were from the local colony (Sprague-Dawley strain). Young were from females fed the following ration: ground yellow corn, 55.5%; soybean meal, 20; alfalfa leaf meal, 2; dried skim milk, 20; condensed fish solubles, 1; dried brewers yeast, 1; salt, 0.5; calcium carbonate, 0.5 and cod liver oil, 0.5. At weaning, the young were fed a basal ration of the following composition for a period of 10 days: soybean protein preparation (sodium proteinate, Archer-Daniels-Midland Company, Minneapolis, Minn.) to provide 20% protein; salts (Hegsted, *et al., J. Biol. Chem.,* 1941, v138, 460), 4.0; cottonseed oil, 5.0 and corn starch to make 100%. The following vitamins were added per kg of ration (excessive levels were maintained to provide for any increased need): thiamine, 8 mg; riboflavin, 12; pyridoxine, 6; calcium pantothenate, 40; nicotinic acid, 40; inositol, 40; p-amino benzoic acid, 40; pteroylglutamic acid, 2; choline chloride, 2 g. In addition, vitamins A and D and alpha-tocopherol were administered by dropper twice weekly. The thyroid-active material was omitted during this period, because, in our experience, more uniform response has been noted in rats weighing from 80-100 g than in smaller animals. For the ensuing 2 weeks the animals were allowed access to the above basal ration modified to contain 0.25% iodinated caesin (Protamone, Cerophyl Laboratories, Kansas City, Mo.). Supplements were added to this ration on the basis of nitrogen equivalence, replacing sodium proteinate.

The results of such supplementation are presented in Table I. These data represent the combined results of eleven separate trials with nine different supplements. Some supplements have been used repeatedly; others have been tested only once or twice. The growth of males and females during the trial period are presented separately, because we have frequently observed a sex difference in response to supplementation. It is recognized that interpretation of the results is rendered more difficult by presentation in such a condensed manner, but limitations of space will not permit tabulation of the results of each of the trials. These detailed results are summarized in the thesis of Bolene(7).

Before discussing some of the implications of these data, it seems well to point out that in our experience rats of the same strain obtained from another source (a commercial dealer) did not respond in the same manner. They were less sensitive to the growth-depressing effect of iodinated casein and did not respond to supplementation in like manner when the level of thyroxine-like materials was increased. It may be that the stock ration fed

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3. Frost, D. V., Fricke, H. H., and Spruth, H. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 102.

4. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 209.

5. Watts, A. B., Swank, W., Ohman, R. J., Ross, O. B. and MacVicar, R. W., *J. Nutrition,* 1950, v41, 533.

6. Lewis, U. J., Tappan, D. V., Register, U. D. and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 568.

7. Bolene, Charles, *Studies on Antithyrotoxic Factors,* Thesis, Oklahoma A. and M. College, 1950.

TABLE I. Effect of Supplementation on Growth of Rats Receiving Toxic Levels of Iodinated Casein.

Supplement	Level in ration %	No. of animals	Avg gain per rat for 2 wk exp. period	Avg % change with respect to basal[1]
		Males		
None	——	28	29.9	——
Fish solubles	1.5	6	44.8	77.7**
APF No. 3 (Merck)	0.5	10	48.8	50.1**
APF supplement (Lederle)	2	6	27.2	60.3[2]
1:20 liver powder (Wilson)	2	17	49.7	92.6**
Biopar A (Armour)	3	5	35.6	109.9[2]
Cryst. B-12	25 mcg/kg	21	45.0	50.7**
Liver residue	3	10	44.4	120.6**
Extracted liver residue	3	5	43.0	35.2[2]
Extract of liver residue	3	4	45.2	42.3[2]
		Females		
None	——	32	30.8	——
Fish solubles	1.5	4	47.9	48.7[2]
APF No. 3 (Merck)	0.5	8	46.1	39.1*
APF supplement (Lederle)	2	12	32.8	28.3
1:20 liver powder (Wilson)	2	11	43.0	51.7**
Biopar A (Armour)	3	12	24.1	11.6
Cryst. B-12	25 mcg/kg	24	33.6	13.6
Liver residue	3	20	42.4	38.5**
Extracted liver residue	3	4	30.8	14.1[2]
Extract of liver residue	3	5	39.6	10.5[2]

[1] Comparisons made with respect to the basal ration in each trial in which supplement appeared, weighed for disproportionate numbers.

[2] Not subjected to statistical analysis due to small numbers.

* and ** indicate statistical significance by analysis of variance with respect to the basal ration at the 5 and 1% levels of probability.

the females producing the young in our colony provided a lesser amount of the factor antagonizing the growth-depressing effect than was provided by the commercial dealer's stock ration. Transfer of nutrients, (including vit. B_{12}) through the egg to the chick has been repeatedly shown to permit essentially normal growth on rations which are low in the particular factor unless the hens have been previously depleted. It seems likely that an analogous transfer may occur in the case of the factor(s) antagonistic to the growth-depressing effect of thyroid-active agents in the rat.

Examination of the effect of supplementation (Table I) shows that fish solubles, two commercial APF supplements (No. 3, Merck and Co. and Lederle), and 1:20 liver powder (Wilson), materials which have appreciable "animal protein factor" activity for the chick, are also effective in counteracting the growth-depressant effect of toxic levels of iodinated casein. Crystalline vit. B_{12} gave a highly significant growth response in males, but was less effective in females. Even in males, however, the growth response was distinctly less than that obtained with various preparations of liver and fish solubles. This suggests that the deficiency induced by hyperthyroidism is multiple in nature and that vit. B_{12} is one of the components. These findings are essentially in accord with the observations of Betheil and Lardy(2) who used male rats of the same strain employed here. They are at variance with the findings of Ershoff(8) who was unable to demonstrate any "anti-thyrotoxic" effect of vit. B_{12} in rats of the Long-Evans strain fed a basal ration of the sucrose and casein type. If it is assumed that thyroprotein induces a multiple deficiency, of which vit. B_{12} is one component, then the divergence of results may be due either to the presence of some other factor in limited amounts in the rations employed by Betheil and Lardy(2) and by ourselves or to increased

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8. Ershoff, B. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 459.

intestinal synthesis. We are presently unable to suggest any reason for the differences observed between males and females in their response to vit. B_{12}. It may be that there are sex differences in the requirement for some additional factor. This factor could be present in sufficient amounts to permit response on the part of males to vit. B_{12} but so limit the growth of the females that no response to vit. B_{12} supplementation was possible.

The effectiveness of hot-water insoluble liver residue in counteracting the growth depressing effects of thyroid toxicity has been previously observed by Ershoff(4,5). A preparation obtained as a by-product of the manufacture of liver extract (Armour and Company) was tested and found to give a positive response. This substance was more effective in males than in females, although the response with the latter was significant at the 5% level of probability. The hot-water soluble fraction of liver (Biopar A, Armour and Company) was likewise more effective in males than in females but the number of animals was too small for reliability. Very preliminary efforts to fractionate the activity of the liver residue by exhaustive extraction with 70% ethanol yielded inconclusive results. Both the extract and extracted liver residue were moderately active for males and showed little activity in the case of the females. Again, however, the number of animals used has been very limited.

Summary. The growth depressant effect of toxic levels of iodinated casein in the ration of immature male and female rats was partially counteracted by the addition of fish solubles, liver extracts and a commercial APF supplement. Supplementation with crystalline vit. B_{12} was more effective in males than in females, but maximum response was not obtained with this substance. The multiple nature of the "antithyrotoxic factor" is therefore suggested.

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Course of Experimental Tuberculosis in the Albino Rat as Influenced by Cortisone.* (18282)

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(Introduced by Paul B. Beeson)

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Previous studies in this laboratory have demonstrated certain quantitative differences in the cellular response of the rat and the rabbit to the intraperitoneal injection of mineral oil(1). It was shown that an oil-induced peritoneal exudate is strikingly different in the rabbit, naturally susceptible to tuberculosis, and in the rat, naturally resistant to the disease. The rat responded to the intraperitoneal injection of mineral oil with an exudate richer in total cells, percentage of mononuclear cells, and in the absolute number of mononuclear cells than did the rabbit. The administration of cortisone depressed the total exudate in both species with the total and differential counts of the rat approaching those of the normal rabbit(2). These results suggested that tuberculosis in the naturally resistant rat might be altered by cortisone administration. The albino rat responds to infection with virulent human tubercle bacilli with a chronic, granulomatous, non-fatal process in which caseation is inconspicuous(3). Indeed, in some strains no gross or microscopic lesions appear to develop, despite the fact that tubercle bacilli can be cultured from the tissues and blood as long as 120 days after inoculation(4).

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* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Bloom, W. L., Cummings, M. M., and Michael, M., Jr., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 171.

2. Cummings, M. M., Michael, M., Jr., Bloom, W. L., in press.

We are reporting herewith the results of experiments designed to test the effect of cortisone on resistance to tuberculosis in the albino rat.

Materials and methods. Albino male Sprague-Dawley rats, whose average weight was 150 g, were used in the studies. These animals were free of bartonella and *P. pseudotuberculosis* infection. Two experiments were carried out at different times. In the first experiment, 15 rats were injected intraperitoneally with 2 mg of a young culture of *M. tuberculosis* (H37Rv). Ten of the animals were given cortisone, 5 mg daily in a single injection for the duration of the experiment. The remaining untreated animals in this group served as controls. Five uninfected animals received 5 mg of cortisone daily throughout the study. In the second experiment, the infecting dose of tubercle bacilli was 4 mg. Ten rats were given cortisone alone, 10 received cortisone plus tubercle bacilli, and 20 were infected only with tubercle bacilli. Of this latter group, 10 served as controls for survival times and the other group as controls for pathologic studies of the infected and treated animals.

The first experiment was terminated at 42 days and the second at 52 days. In the second study, with the death of each animal infected and treated with cortisone, a control infected animal was killed. Sections of lungs, liver, spleen, and adrenals were fixed in formalin and in Zenker's solution for staining with Ziehl Neelson and hematoxylin eosin.

Results. The survival time for all groups of animals is indicated in Table I. It will be noted that at the end of the first experiment 100% of the infected animals survived. One animal was killed at 24 days for pathologic studies. Of 5 animals given cortisone alone, 80% survived. One rat was killed at 24 days. On the other hand, of the group infected and given cortisone, only 10% were living at the conclusion of the study period. In the second experiment, the results were almost identical. One hundred percent of the group given tubercle bacilli survived, 90% of the group given cortisone alone survived, whereas only 30% of those infected with tubercle bacilli and "treated" with cortisone survived the 52 days of the experiment. The control animals, *i.e.*, those receiving either cortisone alone or tubercle bacilli alone, were still living one month after the study was concluded. Combining survival times for both studies, the figures are as follows: tubercle bacilli alone, 100%; cortisone alone, 87%; and tubercle bacilli plus cortisone, 20%. This significantly higher mortality rate for the infected group receiving cortisone far exceeds that previously recorded for rats infected with tubercle bacilli.

TABLE I.
Survival Times for All Groups of Animals in Each Experiment.

No. of animals	Group	Day of death	No. survivors at 42 days
		Experiment 1	
5	TB	24*	4
5	Cortisone	24*,27	3
10	TB + Cortisone	16,18,19,21,25, 30,33,36,38	1
		Experiment 2	at 51 days
10	TB		10
10	Cortisone	14	9
10	TB + Cortisone	9,14,19,21,25 42,42	3

* In Exp. 1 denotes animals killed at stated time for pathologic study.

GROSS LESIONS: *Rats infected with tubercle bacilli*: In all animals, the spleen was enlarged. Diffuse, small, pearly nodules were seen on the pleural surfaces of the lungs in the animals killed after the first month of infection. The lungs otherwise appeared grossly normal. Cultures made from the lungs of several animals in this group were positive for tubercle bacilli.

Rats uninfected, but given cortisone: The spleens were small and no pulmonary lesions were noted. In one animal from Exp. 1 killed on the 40th day, a draining abscess was

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3. Cobbett, L., The Causes of Tuberculosis; Cambridge, Mass., 1917.

4. Ornstein, G. G., and Steinbach, M. M., *Am. Rev. Tub.*, 1925, v12, 77.

present, extending from the right inguinal region for 2 cm into the right flank. *Staphylococcus aureus* was cultured from this abscess. The adrenals of all rats in this group were quite small. Tubercle bacilli were not recovered in cultures made of the lungs of several animals in this group.

Rats infected with tubercle bacilli and given cortisone: The spleens of animals in this group appeared normal in size. The lungs of three rats in the first experiment (dying on the 19th, 25th, and 30th days) were studded with large, caseous masses involving whole lungs and even invading the chest walls in one instance. Similarly, 4 animals in the second experiment (2 being killed on the 51st day at termination and one each dying on the 25th and 42nd days) showed these lesions. We were unable to isolate *P. pseudotuberculosis* from any of these caseous masses. The lungs of the remainder of the rats showed scattered, purplish, gelatinous, atelectatic areas, at times involving an entire lobe, but usually remaining patchy. There was no correlation between duration of infection and appearance of the lesion. Cultures made from various organs of animals selected at random were positive for tubercle bacilli.

MICROSCOPIC LESIONS: *Rats infected with tubercle bacilli*: The lungs of these animals showed clusters of pale, mononuclear cells, surrounded in some areas by lymphocytes and with little or no alveolar exudate or surrounding edema. The whole picture was that of an orderly well-contained granulomatous process. Acid-fast stains showed a few organisms located chiefly within the mononuclear cells comprising the tubercles. The spleens contained small collections of epithelioid cells in the center of Malpighian corpuscles. Acid-fast bacilli were sparse, located principally within these cells. No lesions were noted in the liver and only small numbers of acid-fast organisms were found in the Kupffer cells.

Rats uninfected, but given cortisone: The histologic changes in this group were those expected from prolonged cortisone administration. The only microscopic findings in the lungs were areas of pulmonary edema and hemorrhage probably resulting from killing the animals by a blow on the head.

Rats infected with tubercle bacilli and given cortisone: In contrast to the lungs of the group infected, but given no cortisone, these animals had diffuse interstitial pneumonitis with profuse alveolar exudation containing large numbers of mononuclear cells, but a paucity of lymphocytes. There was no attempt at tubercle formation and all areas of lung tissue were involved with the pneumonitis. The lungs of the rats described as having caseous areas showed in addition to the described mononuclear lesions intense polymorphonuclear exudation in the alveoli. Acid-fast stains revealed numerous bacilli throughout the lung appearing in the exudate and in interstitial areas. The organisms were much more plentiful than in the group not receiving cortisone. The spleens showed the depletion of lymphoid elements; a few Malpighian corpuscles remained. Numerous acid-fast bacilli were found in the supporting stroma. The livers showed abundant acid-fast organisms lying principally within the Kupffer cells.

Discussion. Our previous studies have shown that cortisone depresses an inflammatory exudate which is characteristic of the cellular response to mineral oil. This exudate is quite similar to that stimulated by tubercle bacilli. Quantitative differences in this mineral oil inflammatory reaction can be demonstrated in the naturally resistant and naturally susceptible animal. Thus, a quantitative depression of the inflammatory response to lipids might reduce the natural resistance of the host to tubercle bacilli if it can be said that cellular defenses are important in this infection. This seems to be borne out by the present series of observations. The changes in the cellular response induced by cortisone may not only be suppression of the over-all capacity of the host response; but, also, a more specific influence on individual cells or on humoral mechanisms. The known specific effects of cortisone on lymphocytes(5), neutrophils(6), and fibroblasts(7), could disturb the orderly response to infection with tubercle bacilli and result in a more favorable

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5. Dougherty, T. F., and White, A., *Am. J. Anat.*, 1944, v77, 1.

6. Roche, W. P., and Lathem, W., unpublished observations.

environment for bacterial growth.

Though the mechanism is far from clear, it is apparent from these studies that tubercle bacilli in the rat under the influence of cortisone are able to multiply more rapidly, to produce more diffuse disease, to disseminate, and to result in the death of the animal. It is obvious that findings in an animal resistant to tuberculosis cannot be carried over *in toto* to man, naturally susceptible to tuberculosis. However, the recently recorded(8) adverse effects of cortisone on tuberculosis in the guinea pig, a species naturally susceptible to the infection, would lend support to the suggestion that caution be exercised in considering the administration of cortisone to humans with active tuberculosis.

Summary. 1. Rats injected with virulent tubercle bacilli did not die of tuberculous infection. A majority of rats similarly infected with tubercle bacilli but treated with cortisone died of tuberculosis. 2. Striking histologic differences were noted. Localized granulomatas were seen in the organs of rats injected only with tubercle bacilli, whereas the lesions in the animals also receiving cortisone were diffuse and contained fewer inflammatory cells. 3. The influence of cortisone on experimental tuberculosis appears to be a lowering of the host's natural resistance. The possible modes of action resulting in this are discussed.

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7. Ragan, C., Hornes, H. L., Plotz, C. M., Meyer, K., and Blunt, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 718.

8. Spain, D. M., and Molomut, N., *Ab. Rev. Tub.*, 1950, v62, 337.

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Failure of ACTH or Cortisone to Suppress Tuberculin Skin Reactions in Tuberculous Guinea Pigs.* (18283)

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Some recent reports indicate that tuberculin skin reactions are either obliterated or suppressed following the administration of ACTH or cortisone(1-5). From these observations it has been concluded that the compounds interfere directly with the delayed type of antigen-antibody reaction. Observations made in our laboratories did not confirm the findings reported by others. Therefore, the influence of ACTH or cortisone on the tuberculin skin reaction was tested experimentally.

Material and methods: Fifteen male albino guinea pigs with an average weight of 375 g were injected intradermally with 0.1 ml of 10% Old Tuberculin and were found to be tuberculin negative. The animals were then inoculated subcutaneously in the groin with 0.1 mg of a young culture of human virulent tubercle bacilli (H37 Rv). One month after infection the guinea pigs were divided into 3 groups. Five animals received 2 daily intramuscular injections of 2 mg of ACTH (Armour) for 6 days. Another 5 animals were injected subcutaneously 2 times daily with 2

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* The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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2. Harris, S., and Harris, T. N., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 186.

3. Freeman, S., Fershing, J., Wang, C. C., and Smith, L. C., Proc. 1st Clinical ACTH Conference, edited by J. R. Mote, Blakiston Co., Philadelphia, 1950, p. 509.

4. Favour, C. B., (quoted by Thorne, G. W., *et al.*), *New England J. Med.*, 1950, v242, 866.

5. Long, J. B., and Favour, C. B., *Bull. Johns Hopkins Hosp.*, 1950, v87, 186.

TABLE I.

		Tuberculin reaction 0.1 ml 10% O.T.			
		24 hr		48 hr	
Exp. group	G.P. No.	Diam. mm	Central blanching	Diam. mm	Central blanching
ACTH	582	15	2+	*	*
4 mg	589	10	3+	*	*
	584	15	+	10	±
	579	10	3+	5	±
	587	10	2+	5	±
Corti-	586	12.5	3+	*	*
sone	588	12.5	3+	*	*
4 mg	596	10	3+	10	±
	591	7.5	2+	5	±
	598	10	+	5	±
Control	576	15	0	*	*
0.5%	597	20	2+	*	*
phenol	578	20	0	10	0
	600	10	0	15	0
	580	20	2+	10	2+

* Sacrificed after 24 hr.

mg of cortisone (Merck) for the same period of time. The remaining 5 guinea pigs served as controls and for six days were given twice daily intramuscular injections 0.2 ml of 0.5% phenol in aqueous solution. On the fourth day of hormone administration tuberculin skin tests were repeated in the same manner as described above. After 24 and 48 hours the diameter and the general characteristics of the skin response were recorded and special attention was given to erythema, induration and central necrosis.

Twenty-four hours after tuberculin skin testing, 2 animals from each group were sacrificed by a blow on the neck. The remaining guinea pigs were killed in the same manner after 48 hours. The site of the skin reaction was delineated and excised in a manner to include the adjacent grossly normal tissues. An autopsy was performed and blocks from the excised skin as well as from lung, liver, spleen, lymph nodes and adrenals were fixed in Zenker's fluid with 5% glacial acetic acid and in 10% USP formaldehyde. Sections were stained routinely with phloxine-methylene blue. Sections from the adrenals were also stained with Scarlet Red.

Results. The results are summarized in Table I. All animals receiving either ACTH or cortisone reacted to tuberculin with definite erythema, induration and central blanching. The blanching was more pronounced in the hormone treated animals than in the controls although these showed unmistakable reactions to tuberculin in every instance. The size of the tuberculin reaction as well as the degree of erythema and induration appeared to be less in the animals receiving hormone.

At autopsy all guinea pigs showed widespread active tuberculous infection with marked involvement of lungs, liver, spleen and lymph nodes.

All animals receiving ACTH or cortisone revealed marked lymphoid depletion of the spleen and lymph nodes. The adrenals of the animals treated with cortisone were about one-third normal size and histologic examination revealed marked cortical atrophy. On gross and histologic examination no obvious difference was noted in the size of the adrenals in the controls as compared with the group treated with ACTH. Histologic studies of the skin at the site of the tuberculin reaction revealed distinct differences in the degree of tissue response between the hormone-treated animals and the controls. Proliferation of fibroblasts and endothelium was less active in the lesions of the former. The suppression of the repair process was noticeable at 24 hours and had become distinct at 48 hours. Differences in the degree of polymorphonuclear leukocytic infiltration were not appreciable.

Discussion. The occurrence of an unmistakable positive tuberculin reaction in all guinea pigs receiving either ACTH or cortisone corresponds with the observations reported by Spain and Molomut(6). Our results differ from the clinical observations and experimental findings of others who have reported obliteration and suppression of the tuberculin reaction following hormone administration. The presence of active progressive tuberculosis in our animals might in part explain this difference since the experimental findings of others were made on animals which were not infected with tuberculosis but merely sensitized with B C G(1,2). Similarly a recent clinical study of the influence of ACTH or cortisone on the tuberculin reaction was

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6. Spain, D. M., and Molomut, N., *Am. Rev. Tuberc.*, 1950, v62, 337.

carried out on patients with diseases other than active tuberculosis(5). We cannot explain the negative tuberculin skin tests reported in patients with active tuberculosis following the administration of ACTH(3,4).

The daily dose of ACTH or cortisone given to the animals in this experiment corresponds to 10.6 mg per kilo body weight. The morphologic changes in the adrenal cortex, the lymphoid tissue and other organs indicate the effectiveness of the injected hormones. Larger doses of cortisone gave similar results. In a different experiment positive tuberculin skin reactions were always present in 20 guinea pigs with active progressive tuberculosis which received daily 5 mg of cortisone for 50 days. In these animals the tuberculin skin reactions were obtained at 30 and 45 days after the beginning of hormone administration.

In our experiments ACTH or cortisone did not obliterate the tuberculin skin reaction but appeared to diminish its intensity. We believe that the hormones do not interfere primarily with the immune mechanism of the delayed type of antigen-antibody reaction. The apparent modification of the reaction might result from the inhibitory effect of adrenal corticosteroids on the acute inflammatory process(7).

Summary. Guinea pigs with active progressive tuberculosis were given ACTH or cortisone for 6 days. A definite tuberculin skin reaction was obtained in all animals during the period of hormone administration. The reaction appeared less marked in the treated animals than in the controls. It is suggested that ACTH or cortisone do not interfere primarily with the immune mechanism of the tuberculin skin reaction but decrease the associated acute inflammatory process.

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The Relationship Between Aureomycin, Chloramphenicol, and Terramycin
(18284)

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Herrell *et al.*(1), working with aureomycin, chloramphenicol and terramycin, found, for a number of strains of *Escherichia coli* and *Aerobacter aerogenes*, that when resistance developed against one of the 3 antibiotics there was a parallel increase in resistance to the other 2.

In the present study, strains of *E. coli* and *Micrococcus pyogenes* var. *aureus 209P* were made resistant to aureomycin or chloramphenicol by subjection to repeated increasing doses of the antibiotics. Their resistance to aureomycin, chloramphenicol and terramycin was then determined. The minimal inhibiting concentrations (M.I.C.) were determined in Difco Penassay Broth according to the method described earlier(2). The results obtained are presented in the accompanying table.

It will be noted that with both organisms, a rise in resistance to either aureomycin or chloramphenicol is accompanied by an increased resistance to the other 2 antibiotics. Thus, after 7 passages in the presence of chloramphenicol, *E. coli* D56 shows a 21-fold increase in resistance to this antibiotic, accompanied by an 11-fold increase towards aureomycin and a 6.5-fold increase to terramycin. After 8 passages in the presence of aureomycin-hydrochloride, a 31-fold increase in resistance is noted, accompanied by 27-fold and 12-fold increases in resistance to chloramphenicol and terramycin-hydrochloride, respectively. The case with *M. pyogenes* var. *aureus 209P* is much the same although re-

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1. Herrell, W. E., Heilman, F. R., and Wellman, W. E., *Conference on Terramycin, Ann. N. Y. Acad. Sci.*, 1950, v53, 448.

2. Donovick, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, v50, 623.

TABLE I.

Organism	Aureomycin HCl MIC in $\mu g/ml$	Chloramphenicol MIC in $\mu g/ml$	Terramycin HCl MIC in $\mu g/ml$
Escherichia coli D56 (Original strain)	0.56	1.88	0.540
E. coli D56 after 5 passages vs. chloramphenicol	2.98	17.0	—
E. coli D56 after 7 passages vs. chloramphenicol	5.36	39.7	3.53
E. coli D56 after 5 passages vs. aureomycin HCl	2.51	5.23	—
E. coli D56 after 8 passages vs. aureomycin HCl	15.6	50.5	6.40
M. pyogenes var. *aureus 209P*	0.0507	1.85	0.122
M. pyogenes var. *aureus 209P* after 9 passages vs. chloramphenicol	0.116	5.57	0.186
M. pyogenes var. *aureus 209P* after 10 passages vs. aureomycin HCl	0.220	4.43	0.312

sistance develops more slowly.

A number of reports have appeared which suggest that an increase in resistance to an antibiotic may be paralleled by a rise in resistance to related antibiotics. Donovick and Rake(3) showed that 2 species of bacteria resistant to streptomycin were also resistant to dihydrostreptomycin. Rake *et al.*(4) found that when the resistance of a strain of *E. coli* was increased 25-fold for streptomycin there was a similar increase in resistance to mannosidostreptomycin. Further work(5) on the action of the streptomycins versus *Salmonella schottmülleri* has shown that when, by subjection to increasing doses of streptomycin, the organism's resistance is raised 22.6-fold, a parallel rise of 21.3-fold occurs for dihydrostreptomycin, 5.0-fold for mannosidostreptomycin, and 8.0-fold for dihydromannosidostreptomycin. Eisman, *et al.*(6) having made 2 strains of staphylococci resistant to penicillin G found similar rises in resistance to penicillins X and F. Dowling, *et al.*(7) found with various strains of pneumococci, streptococci, staphylococci, and meningococci made resistant to penicillins G and X, that organisms adapted to one penicillin similarly had acquired resistance to the others. Sullivan, *et al.*(8) worked with strains of *S. aureus* and *E. coli* made resistant to streptomycin, streptothricin and penicillin. The streptomycin and streptothricin used were crude extracts of cultures from *Streptomyces griseus* and *Streptomyces lavendulae*. They found that *E. coli* which had developed resistance to streptothricin was also more resistant to streptomycin. The reverse, however, was not true.

In so far as cross resistance to different antibiotics is concerned, the findings of Kelner and Morton(9) are difficult to interpret. These authors in describing the characteristics of lavendulin and actinorubin, noted that a 26,600-fold increase in resistance of *E. coli* P216, to streptomycin was accompanied by insignificant increases in resistance to lavendulin, actinorubin, and streptothricin. However, when the sensitive parent strain was made resistant to actinorubin by a factor of 64, it was also found to be 16 times more resistant to streptomycin, and 32 times as resistant to streptothricin and lavendulin. Similarly, when the sensitive parent strain was made resistant, by a factor of 32, to lavendulin, this organism had also become equally resistant to the 3 other antibiotics. Thus, while lavendulin and actinorubin had bacterial spectra different from each other, as well as different from those of streptomycin and streptothricin, yet under proper conditions, cross resistance appeared to occur. Since the purities of the various antibiotics used were not specified it is not possible to determine whether or not these cross resistances indicated similar modes

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3. Donovick, R., and Rake, G., *J. Bact.*, 1947, v53, 205.

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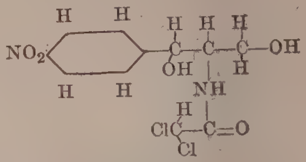
8. Sullivan, M., Stahly, G. L., Birkeland, J. M., and Myers, W. G., *Science*, 1946, v104, 397.

9. Kelner, A., and Morton, H. E., *J. Bact.*, 1947, v53, 695.

of action, or simply the presence of more than one antibiotic in the materials employed for developing the resistant strains. From the results obtained by Kelner and Morton(9), one might expect cross-resistance to develop against streptomycin and streptothricin, as had already been reported by Sullivan, *et al.* (8). However, Smith(10) has described a gram-negative organism which was resistant to 16,000 units of streptomycin per ml while it was inhibited by 7.5 units of streptothricin per ml. Hence, the picture on cross resistance, as far as these 2 antibiotics are concerned, is far from clear.

It would seem to be reasonable to assume that the occurrence of cross resistance of an organism between 2 antibiotics might well indicate that the 2 substances interfere with the same biological processes. This would not be surprising with the various penicillins or streptomycins. It would be more so in the case of streptomycin and streptothricin, but this observation remains somewhat in doubt. Therefore, the occurrence of cross resistance of organisms to chloramphenicol, aureomycin and terramycin is of particular interest. The chemistry of the first of these is completely known and has been described(11), but such is not the case with the latter two. Nevertheless, the empirical formulae of all three are known and indicate that the 3 antibiotics are distinct, one from the other, chemically(12-15).

Chloramphenicol $C_{11}H_{12}N_2O_5Cl_2$



Aureomycin $C_{23}H_{27}N_2O_9Cl$

Terramycin $C_{22}H_{24-26}N_2O_9$

Judging from the absorption spectra of aureomycin and terramycin, these 2 antibiotics must have much in common chemically. Their bacterial spectra are also similar.

Of great interest to the biologist would be the occurrence of cross resistance of an organism to compounds differing from each other fundamentally. This cannot be said to be the case, for example, where the streptomycins or the penicillins are involved. As already indicated, there is reason to believe that the case of cross resistance of organisms between streptomycin and streptothricin may be in doubt. Until complete descriptions of the molecular structures of aureomycin and terramycin become available, one cannot conclude the cross resistance shown to develop here for chloramphenicol, terramycin and aureomycin is an example of cross resistance to distinct chemical types of compounds.

Summary. Escherichia coli D 56 and *Micrococcus pyogenes* var. *aureus 209P* on becoming resistant to aureomycin also become resistant at somewhat lower levels to chloramphenicol and terramycin. Similarly, on becoming resistant to chloramphenicol the two organisms become resistant at lower levels to aureomycin and terramycin.

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Congenital Japanese B Encephalitis Infection of Swine. (18285)

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In Japan a large number of stillbirths was reported among swine in various stages of gestation during the summer months of 1947 and 1948. This was especially prevalent during August and September, with a morbidity approximating 60 to 70% of the pregnant animals. The disease was observed from northern Hokkaido to Kyushu, the southernmost island of Japan. A majority of the pregnant females delivered their dead young at or near the expected date of delivery. In some instances, live, dead and moribund piglets were observed in the same litter. Concomitantly, there occurred among equidae and humans an outbreak of Japanese B encephalitis, which was one of the largest ever recorded (1948).

Through questioning, it was ascertained that a disease among swine with comparable symptomatology occurred during the great epidemic of Japanese B encephalitis in Japan during 1935. No published report is available. The high incidence of inapparent infection with the virus of Japanese B encephalitis has been demonstrated among swine in a survey of antibodies for this agent. These data and the simultaneous occurrence of epidemics in swine, horses and humans suggested the possibility of an interrelationship(1).

In this study a neurotropic virus was isolated from typical swine cases and was identified as the virus of Japanese B encephalitis.

General aspects. Various stages of fetal development of the aborted animals were observed in a given litter. Mummified fetuses, together with normally developed ones, were observed. Subsequent to delivery, most of the young which appeared normal in development were weak and unable to stand; to a lesser extent some, which appeared normal at birth, later developed symptoms of central nervous system involvement. At autopsy, a high percentage of the young had either an encephalomalacia or internal hydrocephalus with the cerebral cortex appearing extremely thin in some cases. Histopathologically, a nonpurulent encephalitis was found among a number of cases. A large amount of exudate was observed in the pleural, pericardial or peritoneal cavities. In contrast to the offspring, no abnormalities were observed among the maternal animals, although some owners noticed anorexia and a slight fever of short duration occurring at varying periods of pregnancy.

Isolation of virus. Materials. During the last week in July, 1948, a litter of 5 pigs was delivered near Hachioji, a city in Tokyo Prefecture. One of the newborn was delivered moribund; 3 of the pigs were too weak either to stand or nurse and expired within 48 to 72 hours. The remaining animal appeared normal in size and development and grew through adolescence without any apparent abnormalities.

The 3 affected piglets were autopsied immediately after death, and fragments of brain were preserved in glycerine saline solutions for biological study (V-4948, V-4949 and V-4950).

Pathologic anatomy. Macroscopic findings. Subcutaneous tissues showed advanced evidence of edema with the skeletal muscle fibers having a turbid appearance. The thoracic and mesenteric lymph nodes showed inflammatory swelling. A large amount of reddish colored fluid was observed in the thoracic and abdominal cavities. Serous membranes contained petechial hemorrhages. Multiple small areas of necrosis were present in the liver and spleen. The stomach contained a small amount of yellow stringy material. The meninges were intensely congested and the encephalon was edematous and extremely soft and friable. On sectioning, the encephalon was hyperemic with numerous extravasations of blood. Congestion of the spinal cord

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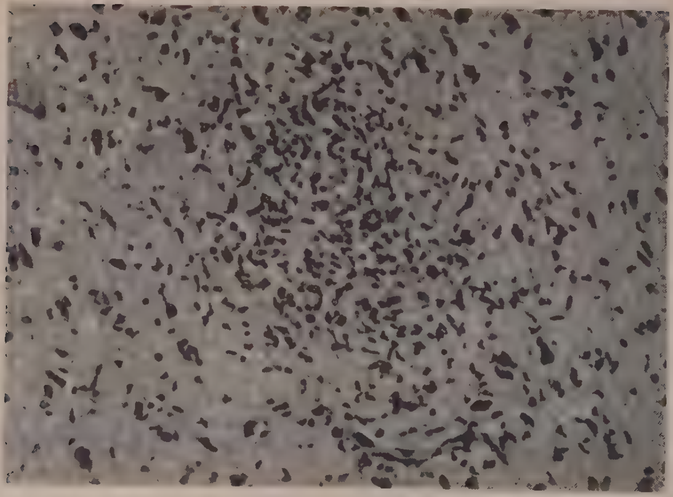

FIG. 1.
Subcortical area showing glial nodule. H and E. ×260.

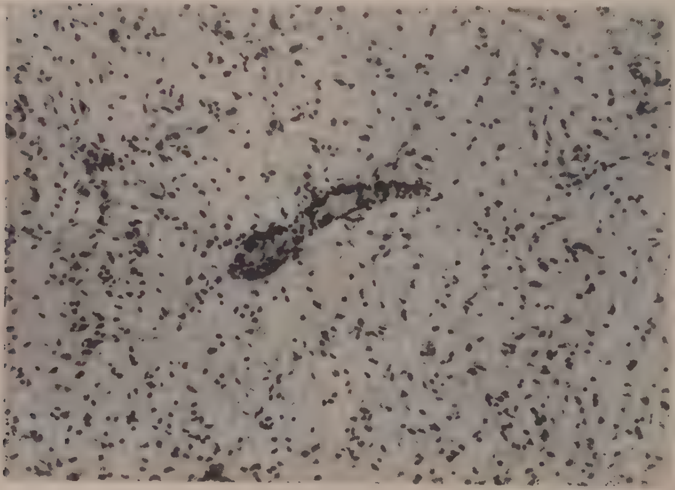

FIG. 2.
Cerebral peduncle showing perivascular cuffing and interstitial infiltration. H and E. ×160.

was also noted. Small areas of softening in the substance of the brain and in the walls of the ventricles were present. The subarachnoid space was filled with a slightly turbid fluid.

Microscopic findings. Histopathologically, changes seen were typically those associated with a virus encephalitis (Fig. 1 and 2), and were diffusely distributed throughout the cortex, white matter, basal ganglia and brain stem. In scattered areas neurones showed changes ranging from those of slight acute cell degeneration to advanced destruction with karyolysis and cytolysis with neuronophagia. There were also numerous small vessels throughout the tissues showing perivascular

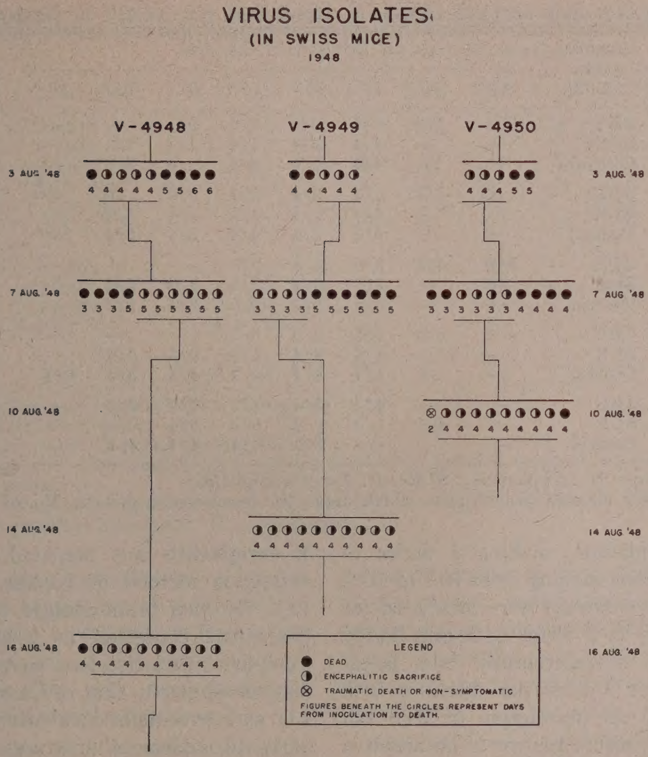

FIG. 3.

cuffing with round cells. Focal areas of glial nodules composed of proliferating microglia and round cells were scattered through both white and gray substance. In the latter area, remnants of degenerated neurones could occasionally be identified.

Isolation of filterable agent. Within 24 hours subsequent to autopsy, each glycerinated brain specimen (V-4948, V-4949 and V-4950) was ground in a mortar and a 10% emulsion prepared, using as diluent 10% rabbit serum in saline. This suspension was centrifuged at 3000 r.p.m. for 15 minutes. The supernate was inoculated intracerebrally in 0.03 ml amounts into 5 to 10 two-week-old mice. Two strains of white mice were used for each specimen; one group was a Bagg-Swiss strain procured in the United States and reared under screen. The second group of mice was purchased locally from Saitama Prefecture animal farms, located approximately 40 miles north of Tokyo. Japanese B encephalitis is considered to be endemic in this area.

Results. The results of isolations in Swiss mice were similar to those obtained in Saitama mice. Both groups of mice (Saitama and Bagg-Swiss) of the first passage exhibited definite signs of encephalitis within a 3 to 6 day incubation period. Subsequent serial passages were readily accomplished by intracerebral inoculation of the infected brain emulsions (Fig. 3). Brain tissue of passage mice was examined histologically and revealed a nonpurulent encephalitis.

Neutralization tests. Intracerebral neutralization tests were carried out with 0.2 ml volumes of undiluted and unheated serum against an equal amount of 3 or 4 ten-fold dilutions of brain suspensions from mice infected with the swine isolates and with viruses of Japanese B encephalitis (Nakayama) and

TABLE I. Identification of Virus Isolates as Japanese B Encephalitis by Neutralization Tests.

Virus	Immune serum (rabbit)	Dilutions of the virus								Log LD$_{50}$	Neutralization log index
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
V-4948	JBE*	—	1/4†	0/4	0/4	0/2	0/4	—	—	2.75	5.0
	SLE*	—	—	4/4	2/4	1/4	1/4	1/4	—	5.75	2.0
	Control	—	—	4/4	4/4	4/4	2/4	3/4	—	7.75	—
V-4949	JBE	—	2/4	0/4	0/4	0/4	0/4	—	—	3.0	4.0
	SLE	—	—	4/4	2/4	1/4	1/4	1/4	—	5.75	1.25
	Control	—	—	4/4	2/4	4/4	2/4	2/4	—	7.0	—
V-4950	JBE	4/4	4/5	3/5	2/4	0/5	—	—	—	4.4	2.8
	SLE	—	—	5/5	4/4	5/5	4/5	2/4	—	7.8	<1.0
	Control	—	—	5/5	5/5	5/5	2/5	1/3	—	7.2	—
JBE (Nakayama)	JBE	4/4	5/5	5/5	1/5	0/5	—	—	—	4.6	3.65
	SLE	—	—	4/4	4/4	2/4	0/3	0/4	—	6.0	2.25
	Control	—	—	4/4	4/4	4/4	4/4	3/4	0/4	8.25	—
SLE	JBE	—	—	4/4	3/4	1/3	1/4	2/3	—	6.5	2.0
	SLE	—	4/4	1/4	0/4	0/3	0/4	—	—	3.75	4.75
	Control	—	—	4/4	3/3	4/4	4/4	4/4	—	8.5	—

* JBE—Japanese B encephalitis; SLE—St. Louis encephalitis.

† The numerator denotes No. of mice which died; the denominator denotes No. of mice inoculated.

St. Louis encephalitis, making a series of final virus dilutions ranging from 10^{-4} to 10^{-8}. The serum-virus mixtures were incubated for 2 hours at 37°C in a water bath and finally were inoculated intracerebrally into Bagg-Swiss mice, 4 or 5 mice per dilution, each mouse receiving an inoculation of 0.03 ml. A normal rabbit serum known to be negative for Japanese B virus was included as a control in each test. The neutralization log index, representing the ratio between LD$_{50}$ end-point of the control and the immune serum, was calculated in the usual manner. Tabulated results are shown in Table I.

Complement fixation tests. Antigens of isolates, V-4948, V-4949 and V-4950, and St. Louis encephalitis (SLE) were prepared by high speed centrifugation. Ten percent mouse brain suspensions in physiologic saline containing 2% inactivated serum from normal guinea pigs were centrifuged horizontally at 2000 r.p.m. for 30 minutes after over-night incubation in the refrigerator. The supernates, subsequent to freezing and thawing, were then centrifuged at 15,000 r.p.m. for one hour in the angle head of an "International" centrifuge under refrigeration with solid CO_2. To the clear supernates, merthiolate was added to give a final concentration of 1:10,000. These were stored at 5°C. Antigen of the Nakayama strain of Japanese B encephalitis was prepared by the benzol extraction method of Espana and Hammon (2). Normal brain antigen for control purposes was processed in a similar manner. Complement fixation tests were performed in a manner similar to that of Casals and Palacios (3) with over-night incubation of the serum-antigen-complement mixtures, and complement titrations in the presence of antigens at 5°C. Four units of virus antigen contained in 0.25 ml were used in the test. Immune guinea pig serum was employed routinely as a positive control. Results are summarized in Table II.

Discussion. The occurrence of an increased incidence of stillbirths and death of newborn in swine populations has been observed previously, but to the best of my knowledge its relationship to Japanese B encephalitis has not been suggested. The correlation between epidemics of the disease and equine epizootics has been recently noted(4-6). Meiklejohn *et al.*(7) reported the experimental infection with Japanese B enceph-

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TABLE II. Identification of Isolates as Japanese B Encephalitis Virus by Complement Fixation Tests.

Immune sera	Antigens	Dilutions of immune serum									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	NMB*	Sal.*
JBE Nak.*	V-4948. U	—	—	—	4	4	4	±	0	0	0
Guinea pig	V-4949. U	—	—	—	4	4	4	±	0	0	0
56°C, 20 min.	V-4950. U	—	—	—	4	4	4	±	0	0	0
	JBE Nak. (1:16)	—	—	—	4	4	4	4	3	0	0
	SLE* (1:2)	—	—	—	0	0	0	0	0	0	0
SLE	V-4948. U	3	±	0	0	0	0	—	—	0	0
Mouse	V-4949. U	3	±	0	0	0	0	—	—	0	0
60°C, 20 min.	V-4950. U	3	±	0	0	0	0	—	—	0	0
	JBE Nak. (1:16)	2	0	0	0	0	0	—	—	0	0
	SLE (1:2)	4	4	3	0	0	0	—	—	0	0

* JBE Nak.—Japanese B encephalitis, Nakayama strain.
SLE—St. Louis encephalitis.
NMB—Antigen control: normal mouse brain.
Sal.—Mixture with physiological salt solution instead of antigen to check on anticomplementary properties of the serum.
Complement fixation is regarded as 4; different degrees of partial fixation as 3, 2, or 1. ±, trace. 0—No fixation or complete hemolysis. U = Undiluted.

alitis virus of 3 pigs, approximately 4 months of age with the production of fatal encephalitis in 2 of the 3 animals following the intravenous injection of the virus. It is of interest to note that they were unable to recover the virus from the dead pigs.

The concurrence of this disease in swine and the Japanese B encephalitis epidemic of 1948 suggested an identical etiologic agent. Histopathologic study and virus isolates confirm this viewpoint.

It is believed that this is the first demonstration of congenital damage of the nervous system by any of the arthropod-borne encephalitis viruses.* During recent years the subject of congenital damage to the nervous system by various infectious agents has become important in medicine. The effects of congenital infection with German measles virus and toxoplasma in human beings have received special consideration in recent years. In human congenital toxoplasmosis, the situation is similar to that described in swine in the present communication, namely, the mother shows either no or very negligible evidence of infection, while the central nervous system of the fetus is severely affected.

Summary. 1. A congenital disease of swine is reported which results in stillbirth or death during the neonatal period. The chief pathologic changes are those of a nonbacterial encephalitis. 2. This disease showed a temporal relationship to epidemics and equine epizootics of Japanese B encephalitis. 3. A virus identified as that of Japanese B encephalitis was recovered from the brain of 3 piglets which died 48 to 72 hours after birth. 4. Congenital damage of the nervous system by an arthropod-borne encephalitis virus has thus been demonstrated in swine.

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